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SPECIFICATION

Screening Method Using CD100

Technical Field

The present invention relates to a screening method for compounds or salts thereof that are useful as antibody production inducers or as agents for the prevention or treatment of diseases caused by abnormal antibody production characterized by using CD100 (Proceedings of the National Academy of Sciences of the USA (Proc. Natl. Acad. Sci. USA), Vol. 93 (1996), pp. 11780-11785, etc.) or salts thereof and receptors thereof and CD72 (Journal of Immunology (J. Immunol.), Vol. 149 (1992), pp. 880-886, etc.).

Background Art

B cells can produce any one of five types of antibodies comprising IgM, IgD, IgG, IgA, and IgE. IgM is produced first in terms of gene structure when the B cells differentiate and first encounter an antigen in vivo. However, the physiological function of IgM is weaker than that of the other classes of antibodies. When the stimulation by the same antigen continues, the gene is changed and the classes of antibodies other than IgM are produced, thereby actualizing stronger physiological function. This change in immunoglobulin from IgM to other classes is called class switch.

CD40 is a membrane glycoprotein expressed on B cells. It reacts, for example, with CD40L expressed on activated T cells. There is known to be no antibody production, class switch, or vaccine effect in CD40-deficient mice. CD40 is a molecule essential for the antibody production function of B cells. Stimulation of B-cells by CD40 inhibits B cell death by anti-IgM antibody. Stimulation of the B cells by CD40 also induces production of various antibody classes, including IgM. However, it is still unclear how these B cell responses are induced.

Controlling B cell death and class switch should make it possible to regulate B cell antibody production.

Epidemic diseases could be treated effectively if it were possible to rapidly raise the post-vaccination antibody titer to epidemic diseases such as colds and influenza where rapid antibody production is required. However, such a therapeutic method does not currently exist. It would also be an effective means of treating so-called allergies and autoimmune diseases if it were possible to specifically lower the abnormal antibodies in diseases caused by abnormal antibody production such as atopic asthma, atopic dermatitis, rheumatoid arthritis, and allergic rhinitis. However, such as a therapeutic method does not currently exist.

Disclosure of the Invention

The inventors isolated and acquired the gene induced by CD40 and clarified that this molecule is CD100. Furthermore, they elucidated that death of B cell by anti-IgM antibody can be avoided when CD100 forms complexes by binding to CD72 on B cells that have been stimulated by activating factors such as CD40, IL-4 or LPS and that CD100 plays an extremely important role in inducing class switch. When complexes are formed by CD100 binding to CD72 on B cells that have been stimulated by activating factors such as CD40, IL-4 or LPS, class switch is triggered in the B-cells and specific high-affinity antibodies are strongly induced in vivo. These facts demonstrate that substances that induce binding of CD72 and CD100, substances that substitute CD100 and bind to CD72 in place of CD100, substances that heighten binding to CD72 by partial modification of the CD100 molecule, and CD100 itself are effective therapeutic methods for rapidly raising the post-vaccination antibody titer against epidemic diseases, for example, such as colds and influenza.

CD100 also serves as an immunopotentiator against cancer and infections. Substances that inhibit the binding of CD72 and CD100 also serve as effective methods of treating diseases caused by abnormal antibody production, such as atopic asthma, atopic dermatitis,

rheumatoid arthritis, and allergic rhinitis, as they are expected to inhibit only antibody production of activated B cells.

That is, the present invention provides:

(1) A screening method for compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof characterized by using CD100 or salts thereof and CD72 or salts thereof,

(2) A screening kit for compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof characterized by using CD100 or salts thereof and CD72 or salts thereof,

(3) Compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof obtained by using the screening method of (1) above or the screen kit of (2) above,

(4) The compounds or salts thereof of (3) above that promote or inhibit the activity of CD100 or salts thereof,

(5) Pharmaceuticals that contain the compounds or salts thereof of (3) above,

(6) The pharmaceuticals of (5) above that are antibody production inducers or agents to prevent or treat diseases caused by abnormal antibody production,

(7) The pharmaceuticals of (6) above wherein the diseases caused by abnormal antibody production are allergies or autoimmune diseases,

(8) The screening method of (1) above characterized by adding CD100 or salt thereof or CD100 or salt thereof and test compound to cells expressing CD72 and measuring the changes in the antibody levels produced or secreted by the expressing cells,

(9) Nonhuman animals with the CD100 gene knockout and T cell reactivity lost,

(10) A screening method for drugs to prevent or treat diseases caused by CD100 deficiency characterized by using nonhuman animals with the CD100 gene knockout,

(11) A screening method for compounds or salts thereof that change the binding property between CD100 or

salts thereof and receptors thereof characterized by using nonhuman animals with the CD100 gene knockout,

(12) The screening method of (11) above wherein the receptor is CD72 or salt thereof,

(13) Transgenic nonhuman animals with enhanced T cell reactivity characterized by possessing DNA that incorporates an exogenous CD100 gene or mutated gene thereof or the progeny thereof that possess said DNA.

(14) A screening method for drugs to prevent or treat diseases caused by enhanced CD100 characterized by using transgenic nonhuman animals that possess DNA that incorporates an exogenous CD100 gene or mutated gene thereof or the progeny thereof that possess said DNA,

(15) A screening method for compounds or salts thereof that change the binding property between CD100 or salts thereof and receptors thereof characterized by using transgenic nonhuman animals that possess DNA that incorporates an exogenous CD100 gene or mutated gene thereof or the progeny thereof that possess said DNA,

(16) The screening method of (15) above wherein the receptor is CD72 or salt thereof.

Brief Explanation of the Drawings

Figure 1 shows the binding property between CHO cells expressing CD72 and mCD100-Fc in example 1.

Figure 2 shows the IgG1 specific antibody production-promoting activity of CD100 in example 2.

Figure 3 shows the antibody production induction-promoting activity of CD100 in vivo in example 3.

Figure 4 shows the genetic map of the targeting vector used to produce CD100 knockout mice in example 4 and CD100 gene assumed in wild-type mice and knockout mice, CD100 gene structure in wild-type mice and knockout mice, and levels of CD100 protein expression in wild-type mice and knockout mice.

Figure 5 shows the CD5 expression levels in wild-type mice and CD100 knockout mice in example 5.

Figure 6 shows antibody production against TD (T cell-dependent) antigen in example 6.

Figure 7 shows the loss of T cell reactivity in CD100 knockout mice in example 7.

Figure 8 shows the increases in soluble CD100 and autoantibody levels associated with aging in MRL/lpr mice in example 9.

Figure 9 shows the loss of dendritic cell reactivity in CD100 knockout mice in example 10.

Figure 10 shows the enhanced T cell reactivity in CD100 transgenic mice in example 11.

BEST MODE FOR CARRYING OUT THE INVENTION

Specific examples of CD100 in the present invention include not only publicly known CD100 and salts thereof (Proc. Natl. Acad. Sci USA, Vol. 93 (1996), pp. 11780-11785; Journal of Biological Chemistry, Vol. 271 (1996), pp. 33376-33381), but also

(17) Polypeptides characterized by containing the same or substantially the same amino acid sequence as the amino acid sequences shown by SEQ ID NO: 1 or SEQ ID NO: 3 (hereinafter referred to as CD100) or salts thereof or

(18) The CD100 or salts thereof of (17) wherein the polypeptide is a protein that contains an amino acid sequence with from 1 to 30, preferably 1 to 10, amino acids deleted from the amino acid sequence shown by SEQ ID NO: 1 or by SEQ ID NO: 3, amino acid sequence with from 1 to 30, preferably 1 to 10, amino acids added (or inserted) in the amino acid sequence shown by SEQ ID NO: 1 or SEQ ID NO: 3, or amino acid sequence with from 1 to 30, preferably 1 to 10, amino acids substituted by other amino acids in the amino acid sequence shown by SEQ ID NO: 1 or SEQ ID NO: 3.

Specific examples of CD72 in the present invention include known CD72 and salts thereof [The Journal of Immunology, Vol. 144, pp. 4870-4877 (1990); The Journal of Immunology, Vol. 149, pp. 880-886 (1992)]. Mouse CD72 also includes allotypes such as Lyb-$2^{a.1}$, Lyb-$2^{a.2}$, Lyb-2^{b}, and Lyb-2^{c} described in The Journal of Immunology, Vol. 149, pp. 880-886 (1992). Further examples of CD72 include:

(19) Polypeptides characterized by containing the same or substantially the same amino acid sequence as the amino acid sequences shown by SEQ ID NO: 5 or SEQ ID NO: 7 (hereinafter referred to as CD72) or salts thereof or

(20) The CD72 or salts thereof of (19) above wherein the polypeptide is a protein that contains an amino acid sequence with from 1 to 10, preferably 1 to 5, amino acids deleted from the amino acid sequence shown by SEQ ID NO: 5 or SEQ ID NO: 7, amino acid sequence with from 1 to 10, preferably 1 to 5, amino acids have been added (or inserted) in the amino acid sequence shown by SEQ ID NO: 5 or SEQ ID NO: 7, or amino acid sequence with from 1 to 10, preferably 1 to 5, amino acids have been substituted by other amino acids in the amino acid sequence shown by SEQ ID NO: 5 or SEQ ID NO: 7.

The term "substantially the same" in the specification means that the activity of the polypeptide, etc., e.g., binding activity of the ligand (CD100) and receptor (CD72), physiological properties, etc., are substantially the same. The substitution, deletion, addition or insertion of amino acids may not greatly change the physiological characteristics or chemical properties of the polypeptide. In such cases, the polypeptide that has undergone the substitution, deletion, addition or insertion (so-called mutated CD100, mutated CD72, etc.) is said to be substantially the same as that which has not undergone substitution, deletion, addition or insertion. Essentially the same substitutes of the amino acids in said amino acid sequence can be selected from among other amino acids in the class to which this amino acid belongs. Examples of nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. Examples of polar (neutral) amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. Examples of amino acids with a positive electrical charge (basic) include arginine, lysine, and histidine. Examples of amino acids with a negative

electrical charge (acidic) include aspartic acid and glutamic acid.

Methods for manufacturing the CD100 and CD72 used in the present invention will be explained in more detail below.

Examples of the CD100 and CD72 used in the present invention include polypeptides derived from the tissues (e.g., pituitary, pancreas, brain, kidneys, liver, gonads, thyroid, gallbladder, bone marrow, adrenals, skin, muscles, lungs, digestive tract, blood vessels, heart) or cells of humans, warm-blooded animals (e.g., guinea pigs, rats, mice, pigs, sheep, cows, monkeys) and fish. The CD100 may be any as long as it is a polypeptide that contains the same or substantially the same amino acid sequence as the amino acid sequence shown by SEQ ID NO: 1 or SEQ ID NO: 3. The CD72 may be any as long as it is a polypeptide that contains the same or substantially the same amino acid sequence as the amino acid sequence shown by SEQ ID NO: 5 or SEQ ID NO: 7. Examples of substantially the same amino acid sequence as the amino acid sequence shown by SEQ ID NOs: 1, 3, 5 or 7 include amino acid sequences with at least approximately 70%, preferably at least approximately 80%, more preferably at least approximately 90%, even more preferably at least approximately 95%, homology with the amino acid sequence shown by SEQ ID NOs: 1, 3, 5 or 7. For example, examples of CD72 include polypeptides with substantially the same activity as polypeptides that contain the amino acid sequence shown by SEQ ID NO: 5 or SEQ ID NO: 7 in addition to polypeptides that contain the amino acid sequence shown by SEQ ID NO: 5 or SEQ ID NO: 7. Examples of substantially the same activity include ligand binding activity, signal transduction activity, and antibody production capacity. "Essentially the same activity" means that the two substances have substantially the same properties in ligand binding activity, for example. Therefore, intensity or weakness such as the intensity of ligand binding activity and quantitative criteria such as the molecular weight of the polypeptide may differ.

Examples of CD100 include polypeptides with substantially the same activity as polypeptides that contain the amino acid sequence shown by SEQ ID NO: 1 or SEQ ID NO: 3 in addition to polypeptides that contain the amino acid sequence shown by SEQ ID NO: 1 or SEQ ID NO: 3. Examples of substantially the same activity include receptor binding activity and antibody production activity. "Essentially the same activity" means that the two substances have substantially the same properties in receptor binding activity, for example. Therefore, intensity or weakness such as the intensity of receptor binding activity and quantitative factors such as the molecular weight of the polypeptide may differ.

In the CD72 and CD100 in this specification, the left end is the N terminal (amino terminal) and the right end is the C terminal (carboxyl terminal) according to peptide designation custom. For example, the C terminal of polypeptides that contain an amino acid sequence shown by SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, or SEQ ID NO: 7 is usually a carboxyl group (-COOH) or carboxylate ($-COO^-$), but the C terminal may be an amide ($-CONH_2$) or ester (-COOR). Examples of R of the ester include the pivaloyloxymethyl groups commonly used as esters for oral use in addition to C_{1-6} alkyl groups such as methyl, ethyl, n-propyl, isopropyl, and n-butyl, C_{3-8} cycloalkyl groups such as cyclopentyl and cyclohexyl, C_{6-12} aryl groups such as phenyl and α-naphthyl, and C_{7-14} aralkyl groups such as phenyl-C_{1-2} alkyl such as benzyl, phenethyl, and benzhydryl or α-naphthyl-C_{1-2} alkyl such as α-naphthylmethyl.

Salts of physiologically acceptable bases (such as alkali metals, etc.) and acids (organic acids and inorganic acids) are used as the salts of CD72 and CD100 used in the present invention. However, physiologically acceptable acid addition salts are especially preferred. Salts with inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid) or salts with organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid,

tartaric acid, citric acid, maleic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) are used as such salts.

The CD72 and CD100 used in the present invention can be manufactured by methods that accord with the publicly known methods [The Journal of Immunology, Vol. 144, pp. 4870-4877 (1990); The Journal of Immunology, Vol. 149, pp. 880-886 (1992); Proc. Natl. Acad. Sci. USA, Vol. 93 (1996), pp. 11780-11785; Journal of Biological Chemistry, Vol. 271 (1996), pp. 33376-33381], i.e., by purifying the polypeptide from the tissues or cells of humans or warm-blooded animals. They can also be manufactured in accordance with the polypeptide synthesis techniques described below. They can also be manufactured by culturing transformants that contain DNA encoding the polypeptide as described below.

When they are manufactured from the tissues or cells of human, warm-blooded animals, fish, etc., the tissues or cells of the human, warm-blooded animal, or fish, etc., are homogenized, and then extraction by acids or an organic solvent, etc are performed. The polypeptide can then be purified and isolated by submitting said extract to a combination of salting out, dialysis, gel filtration, and chromatography such as reverse-phase chromatography, ion-exchange chromatography, and affinity chromatography.

As mentioned above, the CD72 and CD100 used in the present invention can be manufactured according to publicly known peptide synthesis techniques or by cleaving a polypeptide that contains the polypeptide by a suitable peptidase. The peptide synthesis technique may be either a solid-phase or liquid-phase synthesis technique. That is, the target peptide can be manufactured by condensing partial peptides or amino acids capable of constructing the polypeptide and residual parts, and removing the protective groups when the product has protective groups. Methods (1)-(5) listed below are examples of publicly known methods for condensation and removal of protective groups.

(1) M. Bodanszky and M. A. Ondetti, *Peptide Synthesis*, Interscience Publishers, New York (1966).

(2) Schroeder and Luebke, *The Peptide*, Academic Press, New York (1965).

(3) N. Ezumiya et al., *Peptide Synthesis Fundamentals and Experiments*, Maruzen (1975).

(4) H. Yashima and T. Sakakibara, Biochemical experimentation lectures 1, Protein Chemistry IV, 205 (1977).

(5) H. Yashima (editor), Continued drug development, Vol. 14, Peptide Synthesis, Hirokawa Shoten.

The polypeptide (CD72 or CD100) can also be purified and isolated after reaction by a conventional purification method, i.e., a combination of solvent extraction, evaporation, column chromatography, liquid chromatography, and recrystallization. When the polypeptide obtained by the aforementioned methods is a free compound, it can be converted into a suitable salt by publicly known methods. Conversely, when it is obtained in the form of salt, it can be converted into a free compound by publicly known methods.

Amide forms of CD72 and CD100 can use commercial resins for peptide synthesis suited to amide formation. Examples of such resins include chloromethyl resin, hydroxymethyl resin, benzhydrylamine resin, aminomethyl resin, 4-benzyloxybenzyl alcohol resin, 4-methylbenzhydrylamine resin, PAM resin, 4-hydroxymethyl-methylphenylacetamide methyl resin, polyacrylamide resin, 4-(2',4'-dimethoxyphenyl-hydroxymethyl)phenoxy resin, and 4-(2',4'-dimethoxyphenyl-Fmoc aminoethyl)phenoxy resin. Using such resins, α-amino groups and amino acids with suitably protected side chain functional groups are condensed on the resin through various known condensation methods in accordance with the sequence of the target peptide. The protective groups are removed simultaneously with cutting the peptide from the resin after the end of the reaction and the target polypeptide is acquired by an intramolecular disulfide bond formation reaction in a highly dilute solvent if necessary.

For condensation of the protected amino acids described above, a variety of activation reagents for peptide synthesis may be used, but carbodiimides are particularly preferably employed. Examples of such carbodiimides include DCC, N,N'-diisopropylcarbodiimide, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide, etc. For activation by these reagents, the protected amino acids in combination with a racemization inhibitor (e.g., HOBt, etc.) are added directly to the resin, or the protected amino acids are previously activated in the form of symmetric acid anhydrides, or HOBt esters, followed by adding the thus activated protected amino acids to the resin. Solvents suitable for use to activate the protected amino acids or condense with the resin may be chosen from solvents that are known to be usable for peptide condensation reactions. Examples of such solvents are acid amides such as N,N-dimethylformamide, N,N-dimethylacetamide, N-methylpyrrolidone and others, halogenized hydrocarbons such as methylene chloride, chloroform and others, alcohols such as trifluoroethanol, sulfoxides such as dimethylsulfoxide, amines such as pyridine, ethers such as dioxane and tetrahydrofuran, nitriles such as acetonitrile and propionitrile, esters such as methyl acetate and ethyl acetate, or appropriate mixtures of these solvents. The reaction temperature is appropriately chosen from the range known to be applicable to peptide bond-forming reactions and is usually selected in the range of approximately -20°C to 50°C. The activated amino acid derivatives are used generally in an excess of 1.5 to 4 times. The condensation is examined using the ninhydrin reaction; when the condensation is insufficient, the condensation can be completed by repeating the condensation reaction without removal of the protecting groups. When the condensation is yet insufficient even after repeating the reaction, unreacted amino acids are acetylated with acetic anhydride or acetylimidazole to cancel any possible adverse affect on the subsequent reaction.

Examples of the protecting groups used to protect amino groups of the starting materials include Z, Boc, tertiary-penthyloxycarbonyl, isobornyloxycarbonyl, 4-methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantyloxycarbonyl, trifluoroacetyl, phthaloyl, formyl, 2-nitrophenylsulphenyl, diphenylphosphinothioyl, Fmoc, etc. Examples of the protecting groups for carboxyl group include 2-adamantyl, 4-nitrobenzyl, 4-methoxybenzyl, 4-chlorobenzyl, phenacine groups, benzyloxycarbonyl hydrazide, tertiary-butoxycarbonyl hydrazide, trityl hydrazide in addition to C_{1-6} alkyl groups, C_{3-8} cycloalkyl groups and C_{7-14} aralkyl groups stated above as R.

The hydroxyl group of serine and threonine can be protected by, for example, its esterification or etherification. Examples of groups appropriately used for the esterification include a lower alkanoyl group such as acetyl group, etc., an aroyl group such as benzoyl group, etc., and a group derived from carbon such as benzyloxycarbonyl group and ethoxycarbonyl group. Examples of the group appropriately used for the etherification include benzyl group, tetrahydropyranyl group, tertiary-butyl group, and the like.

Examples of groups for protecting the phenolic hydroxyl group of tyrosine include Bzl, Cl_2-Bzl, 2-nitrobenzyl, Br-Z, tertiary-butyl, etc.

Examples of groups used to protect the imidazole moiety of histidine include Tos, 4-methoxy-2,3,6-trimethylbenzenesulfonyl, DNP, benzyloxymethyl, Bum, Boc, Trt, Fmoc, etc.

Examples of the activated carboxyl groups in the starting material include the corresponding acid anhydrides, azides, activated esters (esters with alcohols (e.g., pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethyl alcohol, p-nitrophenol, HONB, N-hydroxysuccimide, N-hydroxyphthalimide, HOBt)). As the activated form of the amino groups in the starting material, the corresponding phosphoric amides are employed.

To eliminate (split off) the protecting groups, there are employed catalytic reduction in a hydrogen gas flow in the presence of a catalyst such as Pd-black or Pd-carbon; an acid treatment with anhydrous hydrogen fluoride, methanesulfonic acid, trifluoromethanesulfonic acid or trifluoroacetic acid, or a mixture solution of these acids; a treatment with a base such as diisopropylethylamine, triethylamine, piperidine or piperazine; and reduction with sodium in liquid ammonia. The elimination of the protecting group by the acid treatment described above is carried out generally at a temperature of -20°C to 40°C. In the acid treatment, it is efficient to add a cation scavenger such as anisole, phenol, thioanisole, m-cresol, p-cresol, dimethylsulfide, 1,4-butanedithiol or 1,2-ethanedithiol. Furthermore, 2,4-dinitrophenyl group known as the protecting group for the imidazole of histidine is removed by a treatment with thiophenol. Formyl group used as the protecting group of the indole of tryptophan is eliminated by the aforesaid acid treatment in the presence of 1,2-ethanedithiol, 1,4-butanedithiol or the like, as well as by a treatment with an alkali such as a diluted sodium hydroxide solution and dilute ammonia.

Protection of functional groups that should not be involved in the reaction of the starting materials, protecting groups, elimination of the protecting groups and activation of functional groups involved in the reaction may be appropriately selected from publicly known groups and publicly known means.

In another method for obtaining the amides of CD72 and CD100, for example, the α-carboxyl group of the carboxy terminal amino acid is first protected by amidation; the peptide chain is then extended from the amino group side to a desired length. Thereafter, a protein in which only the protecting group of the N-terminal α-amino group has been eliminated from the peptide chain and a peptide (or amino acids) in which only the protecting group of the C-terminal carboxyl group has been eliminated are manufactured. The two

peptides are condensed in a mixture of the solvents described above. The details of the condensation reaction are the same as described hereinabove. After the protected peptide obtained by the condensation is purified, all the protecting groups are eliminated by the method described above to give the desired crude polypeptide. This crude polypeptide is purified by various known purification means. Lyophilization of the major fraction gives the amide of the desired polypeptide.

When obtaining an ester form of CD72 or CD100, an ester form of the desired polypeptide can be obtained in the same way as the amide form of the polypeptide after producing an amino acid ester by condensing the α-carboxyl groups of the amino acid at a carboxyl terminal with the desired alcohol.

The DNA encoding CD72 used in the present invention may be any of the DNA that contains DNA encoding receptor protein comprising the same or substantially the same amino acid sequence as that shown by SEQ ID NO: 5 or SEQ ID NO: 7. The DNA encoding CD72 used in the present invention may be any of the DNA that contains DNA encoding ligand protein comprising the same or substantially the same amino acid sequence as that shown by SEQ ID NO: 1 or SEQ ID NO: 3. It may be any of genomic DNA, genomic DNA library, cDNA derived from the aforementioned tissues or cells, cDNA library derived from the aforementioned tissues or cells, or synthetic DNA. The vectors used in the library may be any of bacteriophages, plasmids, cosmids, or phagemids. It can also be amplified directly by reverse transcriptase polymerase chain reaction (abbreviated hereinafter as RT-PCR) using an RNA fraction prepared from the aforementioned tissues or cells.

More specifically, (1) DNA that hybridizes with sequences that have DNA that contains DNA encoding a receptor protein with an amino acid sequence the same or substantially the same as the amino acid sequence shown by SEQ ID NO: 5 or SEQ ID NO: 7 (or SEQ ID NO: 1 or SEQ ID NO: 3) under stringent conditions and (2) DNA encoding

a polypeptide that has the same amino acid sequence even though it does not form a hybrid with the sequence set forth in (1) and a sequence with DNA that contains DNA encoding a polypeptide that contains an amino acid sequence the same or substantially the same as the sequence shown by SEQ ID NO: 5 or SEQ ID NO: 7 (or SEQ ID NO: 1 or SEQ ID NO: 3) due to degeneration of the genetic code, etc. is used. Hybridization can be performed by or in accordance with known methods. An example of the aforementioned stringent conditions is 42°C, 50% formamide, 4 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA, pH 7.4), 5 × Denhardt solution, 0.1% SDS.

The DNA encoding CD72 or CD100 used in the present invention can be manufactured as well by the following genetic engineering means.

Means of cloning DNA encoding CD72 or CD100 in its entirety include amplification of the target DNA from the aforementioned DNA library, etc., by known PCR using a synthetic DNA primer that has a partial base sequence of the polypeptide or hybridization of DNA incorporated in a suitable vector with one labeled using synthetic DNA or a DNA fragment that contains part or all of the polypeptide region. Hybridization is performed, for example, by the methods described in *Molecular Cloning* (2nd edition: J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989). When using a commercial library, it is conducted by the method described in the attached instructions.

The cloned DNA encoding CD72 or CD100 used in the present invention can be used as is or digested by restriction enzymes with linkers added as desired, depending on the goal. Said DNA may have ATG as a translation initiation codon on the 5' terminal and TAA, TGA, or TAG as a translation stop codon on the 3' terminal. These translation initiation codons and translation stop codons can also be added using appropriate synthetic DNA adapters.

Expression vectors of the CD72 and CD100 used in the present invention can be produced, for example, by (a)

cutting the target DNA fragment from the DNA encoding CD72 or CD100 used in the present invention and (b) ligating said DNA fragment downstream of the promoter in a suitable expression vector.

Plasmids derived from *Escherichia coli* (e.g., pBR322, pBR325, pUC12, pUC13, plasmids derived from *Bacillus subtilis* (e.g., pUB110, pTP5, pC194), plasmids derived from yeast (e.g., pSH19, pSH15), bacteriophages such as λ phage, and animal viruses such as retrovirus, vaccinia virus, and baculovirus are used as vectors. Any promoter can be used as long as it is a suitable promoter that corresponds to the host used in gene expression.

When the host in transformation is an animal cell, a promoter derived from SV40, a retrovirus promoter, metallothioneine promoter, heat shock promoter, cytomegalovirus promoter, SRα promoter, etc., can be utilized. When the host is an organism of the genus Escherichia, a trp promoter, T7 promoter, lac promoter, recA promoter, λPL promoter, lpp promoter, etc., is preferred. When the host is an organism of the genus Bacillus, an SPO1 promoter, SPO2 promoter, penP promoter, etc., is preferred. When the host is yeast, a PHO5 promoter, PGK promoter, GAP promoter, ADH1 promoter, GAL promoter, etc. is preferred. When the host is an insect cell, a polyhedrin promoter, P10 promoter, etc., is preferred.

In addition to the above, those that contain enhancers, splicing signals, poly A addition signals, selection markers, SV40 origin of replication (sometimes abbreviated hereinafter as SV40ori), etc., can be used for the expression vector. Examples of selection markers include a dihydrofolate reductase (sometimes abbreviated hereinafter as dhfr) gene [methotrexate (MTX) resistance], ampicillin resistance gene (sometimes abbreviated hereinafter as Amp^r), and neomycin resistance gene (sometimes abbreviated hereinafter as Neo, G418 resistance). When CHO ($dhfr^-$) cells are used and a DHFR gene used as the selection marker in particular, selection is possible even by thymidine-free medium.

The signal sequence joined to the host if necessary is added on the N terminal of the polypeptide or partial peptide thereof. A PhoA signal sequence, OmpA signal sequence, etc. can be used when the host is an organism of the genus Escherichia. A α-amylase signal sequence or subtilysin signal sequence, etc. can be used when the host is an organism of the genus Bacillus. A mating factor α (MFα) signal sequence or invertase signal sequence, etc. can be used when the host is yeast. Insulin signal sequence, α-interferon signal sequence, or antibody molecule signal sequence, etc. can be used when the host is an animal cell.

Transformants can be produced using vectors that contain DNA encoding CD72 or CD100 constructed in this way.

Organisms of the genus Escherichia, organisms of the genus Bacillus, yeasts, insects or insect cells, animal cells, etc., can be used as the host.

Escherichia coli K12·DH1 [Proc. Natl. Acad. Sci. USA, Vol. 60, 160 (1968)], JM103 [Nucleic Acids Research, Vol. 9, 309 (1981)], JA221 [Journal of Molecular Biology, Vol. 120, 517 (1978)], HB101 [Journal of Molecular Biology, Vol. 41, 459 (1969)], C600 [Genetics, Vol. 39, 440 (1954)], *E. coli* DH10B [Focus 12, p. 19 (1990), D. Lorow et al.], etc., are used as organisms of the genus Escherichia.

Bacillus subtilis MI114 [Gene, Vol. 24, 255 (1983)], 207-21 [Journal of Biochemistry, Vol. 95, 87 (1984)], etc., are used as organisms of the genus Bacillus, etc.

Saccharomyces cerevisiae AH22, AH22R⁻, NA87-11A, DKD-5D, 20B-12 are used as yeasts.

Silkworm larvae, for example, are used as insects [Maeda et al., Nature, Vol. 315, 592 (1985)].

As insect cells, for example a cell line from cabbage looper pupae (*Spodoptera frugiperda* cells; Sf cells), MG1 cells from the midgut of *Trichoplusia ni*, High Five™ cells from *Trichoplusia ni* eggs, cells from *Mamestra brassicae*, and cells from *Estigmena acrea* are used as insect cells when the virus is AcNPV. A silkworm

cell line (*Bombyx mori* N; BmN cells), etc. is used when the virus is BmNPV. Sf9 cells (ATCC CRL1711), Sf21 cells [the above, J. L. Vaughn et al., In Vitro, Vol. 13, 213-217 (1977)], etc. are used as said Sf cells.

Monkey COS-7 cells, Vero cells, Chinese hamster cells CHO, DHFR gene-deficient Chinese hamster cells CHO (dhfr- CHO cells), mouse L cells, mouse 3T3 cells, mouse myeloma cells, human HEK293 cells, human FL cells, 293 cells, C127 cells, BALB3 T3 cells, Sp-2/O cells, mouse B cell line WEHI231 cells, P3U1 plasmacytes, etc., are used as animal cells.

Organisms of the genus Escherichia are transformed, for example, by the methods described in Proc. Natl. Acad. Sci. USA, Vol. 69, 2110 (1972) and Gene, Vol. 17, 107 (1982).

Organisms of the genus Bacillus are transformed, for example, by the methods described in Molecular & General Genetics, Vol. 168, 111 (1979).

Yeasts are transformed, for example, by the methods described in Proc. Natl. Acad. Sci. USA, Vol. 75, 1929 (1978).

Insect cells or insects are transformed, for example, by the methods described in Bio/Technology, Vol. 6, pp. 47-55 (1988).

Animal cells are transformed, for example, by the methods described in Virology, Vol. 52, 456 (1973).

Examples of methods of introducing the expression vector into cells include lipofection [P. L. Felgner et al., Proceedings of the National Academy of Sciences of the United States of America, Vol. 84, p. 7413 (1987)], the calcium phosphate method [F. L. Graham and A. J. van der Eb, Virology, Vol. 52, pp. 456-467 (1973)], and electroporation [E. Neumann et al., EMBO Journal, Vol. 1, pp. 841-845 (1982)].

Transformants transformed by the expression vector that contains the DNA encoding CD72 or CD100 used in the present invention are obtained in this way.

A method of stably expressing the CD72 or CD100, etc. used in the present invention using animal cells is

selection by clone selection of cells that have incorporated the expression vector that was introduced into the aforementioned animal cells into the chromosomes. Specifically, transformants are selected using the aforementioned selection markers as the indicator. Furthermore, stable animal cell lines that have high expression capacity for the polypeptide, etc. can be obtained by repeated clone selection of the animal cells obtained using selection markers in this way. When the dhfr gene is used as the selection marker, animal cell lines with even higher expression can be obtained by intracellular amplification of the DNA encoding the polypeptide or partial peptides thereof together with the dhfr gene by gradually raising the MTX concentration during culture and selecting resistant lines.

The polypeptide, etc. can be manufactured by producing and accumulating the polypeptide, etc. by culturing the aforementioned transformants under conditions that permit expression of the DNA encoding the polypeptide, etc. (CD72, CD100).

A liquid medium is appropriate as the medium used in culture when culturing transformants in which the host is an organism of the genera Escherichia or Bacillus. The medium contains a carbon source, nitrogen source, inorganic material, etc., necessary for the growth of said transformants. Examples of carbon sources include glucose, dextrin, soluble starch, and sucrose. Examples of nitrogen sources include organic and inorganic materials such as ammonium salts, nitrate, corn steep liquor, peptone, casein, meat extract, soybean meal, and potato extract. Examples of inorganic materials include calcium chloride, sodium dihydrogen phosphate, and magnesium chloride. Yeast extract, vitamins, growth accelerators, etc. may also be added. The pH of the medium is preferably approximately 5-8.

M9 medium that contains glucose and casamino acid [Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York, 1972], for example, is preferred as the medium when culturing

organisms of the genus Escherichia. Drugs such as 3β-indolylacrylic acid, for example, can be added if necessary to make the promoter act more efficiently.

When the host is an organism of the genus Escherichia, culture is usually conducted for approximately 3-24 hours at approximately 15-43°C. Ventilation or agitation can also be added as necessary.

When the host is an organism of the genus Bacillus, culture is usually conducted for approximately 6-24 hours at approximately 30-40°C. Ventilation or agitation can also be added as necessary.

When culturing transformants in which the host is a yeast, examples of the medium include Burkholder minimal medium [K. L. Bostian et al., Proc. Natl. Acad. Sci. USA, Vol. 77, 4505 (1980)] and SD medium that contains 0.5% casamino acid [G. A. Bitter et al., Proc. Natl. Acad. Sci. USA, Vol. 81, 5330 (1984)]. The pH of the medium is preferably adjusted to approximately 5-8. Culture is usually performed for approximately 24-72 hours at approximately 20-35°C, with aeration or agitation added as necessary.

When culturing transformants in which the host is an insect cell, medium obtained by appropriate addition of additives such as immobilized 10% bovine serum, etc. to Grace's insect medium (T.C.C. Grace, Nature 195, 788 (1962)) is used as the medium. The pH of the medium is preferably adjusted to approximately 6.2-6.4. Culture is usually performed for approximately 3-5 days at approximately 27°C, with aeration or agitation added as necessary.

When culturing transformants in which the host is an animal cell, MEM medium containing approximately 5-20% fetal calf serum [Science, Vol. 122, 501 (1952)], DMEM medium [Virology, Vol. 8, 396 (1959)], RPMI 1640 medium [The Journal of the American Medical Association, Vol. 199, 519 (1967)], 199 medium [Proceeding of The Society for the Biological Medicine [sic], Vol. 73, 1 (1950)], etc., are used as the medium. The pH is preferably approximately 6-8. Culture is usually performed for

approximately 15-60 hours at approximately 30-40°C, with aeration or agitation added as necessary.

When CHO (dhfr⁻) cells and the dhfr gene as a selection marker are used in particular, it is preferable to use basically thymidine-free DMEM medium that contains dialyzed fetal calf serum.

The CD72 and CD100 used in the present invention can be isolated and purified from the aforementioned culture, for example, by the following methods.

When extracting the CD72 or CD100 used in the present invention from the cultured mass or cells, the mass or cells are collected by a known method after culture, suspended in a suitable buffer, and disrupted by a means such as ultrasonication, lysozyme and/or freezing-thawing. A means of obtaining a crude extract of polypeptide can then be employed, such as centrifugation or filtration. The buffer may contain protein denaturing agents such as urea or guanidine hydrochloride or surfactants such as Triton X-100 (registered trademark, sometimes abbreviated hereinafter as TM).

When the CD72 or CD100 used in the present invention is secreted in the culture broth, the cell mass or cells are separated from the supernatant by a known method after culture has been completed and the supernatant collected.

The CD72 or CD100 used in the present invention contained in the culture supernatant or extract obtained in this way can be purified by an appropriate combination of known isolation and purification techniques. Techniques that utilize solubility such as salting out and solvent precipitation, techniques that utilize primarily differences in molecular weight such as dialysis, ultrafiltration, gel filtration, and SDS-polyacrylamide gel electrophoresis, techniques that utilize differences in charge such as ion-exchange chromatography, techniques that utilize specific affinity such as affinity chromatography, techniques that utilize differences in hydrophobicity such as reverse-phase high-performance liquid chromatography, and techniques that

utilize differences in isoelectric point such as isoelectrophoresis and chromatofocusing are used as these known isolation and purification techniques.

When the CD72 or CD100 used in the present invention obtained in this way has been obtained as a free compound, it can be converted into a salt by or in accordance with known methods. Conversely, when it has been obtained in the form of a salt, it can be converted into a free compound or another salt by or in accordance with known methods.

The polypeptide can also be partially removed by applying arbitrary modifications by causing appropriate protein-modifying enzymes to act before or after purification on the CD72 or CD100 used in the present invention to produce recombinants. For example, trypsin, chymotrypsin, arginyl endopeptidase, protein kinase, glycosidase, etc., are used as protein-modifying enzymes.

The presence of the polypeptide of the present invention produced in this way can be measured by enzyme immunoassay using specific antibodies, etc.

[Screening methods for compounds or salts thereof that change the binding property between CD100 and CD72 (ligand-receptor assay system)]

A screening method for compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof characterized by using CD100 or salts thereof and CD72 or salts thereof and a screening kit for compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof characterized by using CD100 or salts thereof and CD72 or salts thereof (abbreviated hereinafter as screening method of the present invention and screening kit of the present invention) will be described in detail below.

Compounds (e.g., peptides, proteins, nonpeptidic compounds, synthetic compounds, fermentation products, etc.) or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof

can be screened by using CD72 or salt thereof as the receptor or by constructing a recombinant CD72 expression system and using a binding assay system with CD100 or salts thereof (ligand-receptor assay system) that employs said expression system.

Such compounds include compounds that accelerate CD72-mediated immune reactions [e.g., antibody production such as antigen-specific IgG; T cell reactivity such as antigen production to TD (T cell dependent) antigen, T cell growth capacity, IL-4 production, and interferon gamma production; suppression or promotion of dendritic cell reactivity such as IL-12 production, etc.] (i.e., CD72 agonists) and compounds that do not have said immune reaction-promoting activity (i.e., CD72 antagonists).

The term "changes the binding property between CD100 or salts thereof and receptors thereof (e.g., CD72 or salts thereof)" encompasses both inhibition of binding between CD100 or salts thereof and receptors thereof (e.g., CD72 or salts thereof) and promotion of binding with the ligand.

Specifically, the present invention proposes a screening method for compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof characterized by comparing (i) when CD100 or salt thereof is brought into contact with CD72 or salt thereof and (ii) when CD100 or salt thereof and the test compound are brought into contact with the aforementioned CD72 or salt thereof.

Comparison of (i) when CD100 or salt thereof is brought into contact with CD72 or salt thereof as above and (ii) when CD100 or salt thereof and the test compound are brought into contact with CD72 or salt thereof as above is done, for example, by measuring the immune response-promoting activity such as antibody production and the level of ligand binding to said CD72 or salt thereof in the screening method of the present invention.

Specifically, the screening method of the present invention is:

(1) A screening method for compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof characterized by measuring and comparing the level of binding of labeled CD100 or salt thereof to said CD72 or salt thereof when labeled CD100 or salt thereof is brought into contact with CD72 or salt thereof as above and when labeled CD100 or salt thereof and the test compound are brought into contact with CD72 or salt thereof,

(2) A screening method for compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof characterized by measuring and comparing the level of binding of labeled CD100 or salt thereof to said cells or said membrane fraction when labeled CD100 or salt thereof is brought into contact with cells or a membrane fraction of said cells that contains CD72 or salt thereof and when labeled CD100 or salt thereof and the test compound are brought into contact with cells or a membrane fraction of said cells that contain CD72 or salt thereof,

(3) A screening method for compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof characterized by measuring and comparing the level of binding of labeled CD100 or salt thereof to CD72 or salt thereof when labeled CD100 or salt thereof is brought into contact with CD72 or salt thereof expressed on the cell membrane by culturing transformants that contain DNA encoding CD72 and when labeled CD100 or salt thereof and the test compound are brought into contact with CD72 or salt thereof expressed on the cell membrane by culturing transformants that contain DNA encoding CD72,

(4) A screening method for compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof characterized by measuring and comparing the immune response-promoting activity mediated by CD72 or salt thereof [e.g., antibody production such as antigen-specific IgG; T cell reactivity such as antibody production to TD (T cell-

dependent) antigen, growth capacity of T cells, IL-4 production, and interferon gamma production; suppression or enhancement of dendritic cell reactivity such as IL-12 production, etc.] when a compound that activates CD72 or salts thereof (e.g., CD100 or salts thereof) is brought into contact with cells that contain CD72 or salt thereof and when a compound that activates CD72 or salts thereof and the test compound are brought into contact with cells that contain CD72 or salt thereof, and

(5) A screening method for compounds or salts thereof that change the binding property between CD100 or salts thereof and CD72 or salts thereof characterized by measuring and comparing the immune response-promoting activity mediated by CD72 or salts thereof [e.g., antibody production of antigen-specific IgG; T cell reactivity such as antibody production to TD (T cell-dependent) antigen, T cell growth capacity, IL-4 production, and interferon gamma production; suppression or enhancement of dendritic cell reactivity such as IL-12 production, etc.] when a compound that activates CD72 or salts thereof (e.g., CD100 or salt thereof, etc.) is brought into contact with CD72 or salt thereof expressed on the cell membrane by culturing transformants that contain DNA encoding CD72 and when a compound that activates CD72 or salts thereof and the test compound are brought into contact with CD72 or salt thereof expressed on the cell membrane by culturing transformants that contain DNA encoding CD72.

The screening method of the present invention will be explained specifically below.

First, any may be used as the CD72 employed in the screening method of the present invention as long as it contains the aforementioned CD72. Membrane fractions of organs of humans, warm-blooded animals, fish, etc., are appropriate. However, since it is extremely difficult to obtain organs from humans, it is appropriate to use CD72 or salts thereof expressed in large quantities using recombinants as that used in screening.

The method described above and the like are used in the production of CD72 and salts thereof.

When cells or membrane fractions of said cells that contain CD72 or salts thereof are used in the screening method of the present invention, they may be prepared as described below.

When cells that contain CD72 or salts thereof are used, said cells may be immobilized by glutaraldehyde, formalin, etc. Immobilization can be performed by a known technique.

Cells that contain CD72 or salts thereof means host cells expressing CD72 or salts thereof. Examples of said host cells include the aforementioned *E. coli*, *B. subtilis*, yeasts, insect cells, and animal cells.

The membrane fraction means the fraction that contains a large amount of cell membrane obtained by a known method after disrupting the cells. Examples of the means of disrupting the cells include crushing by Potter-Elvehjem homogenizer, disrupting by Waring Blender or polytron (made by Kinematica), disrupting by ultrasonication, and disrupting by spraying the cells from a fine nozzle while pressing by French press, etc. Primarily fractionation by centrifugation such as fractionation centrifugation and density gradient centrifugation are used in fractionation of the cell membrane. For example, the disrupted cell solution is centrifuged for a short time (usually approximately 1-10 minutes) at low speed (500-3000 rpm). The supernatant is then centrifuged for 30 minutes to 2 hours at high speed (15,000-30,000 rpm) and the precipitate obtained taken as the membrane fraction. Said membrane fraction contains large amounts of the expressed CD72 or salt thereof and cell-derived membrane components such as phospholipids and membrane proteins.

The amount of CD72 or salt thereof in said cells or membrane fraction that contains CD72 or salts thereof is preferably 10^3-10^8 molecules per cell, appropriately 10^5-10^7 molecules appropriately. A higher level of expression not only raises the ligand binding activity

relative to the membrane fraction (relative activity) and makes it possible to construct a high-sensitivity screening system, but allows measurement of a greater number of samples in the same lot.

An appropriate CD72 fraction and labeled ligand (CD100) are used to implement the aforementioned methods (1)-(3) to screen compounds that change the binding property between CD100 or salts thereof and CD72 or salts thereof. The CD72 fraction is preferably a natural CD72 fraction or a recombinant CD72 fraction with equivalent activity or the like. Here, the term equivalent activity means equivalent ligand binding activity, etc. A labeled ligand (CD100) or labeled ligand (CD100) analog compound, etc. is used as the labeled ligand. For example, labeled ligands (CD100) labeled by [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc., can be utilized. In particular, labeled CD100 or CD100 derivatives prepared by publicly known methods using Bolton-Hunter reagent can also be utilized.

Specifically, when screening compounds that change the binding property between CD100 or salts thereof and CD72 or salts thereof, a receptor standard is prepared by suspending cells or a membrane fraction of cells that contain CD72 or salt thereof in a buffer appropriate for screening. The buffer may be any that does not inhibit the binding of the ligand and receptor such as pH 4-10 (preferably 6-8) phosphate buffer or Tris-hydrochloride buffer. Surfactants such as CHAPS, Tween-80™ (Kao-Atlas), digitonin, deoxycholate, etc., can also be added to the buffer to reduce nonspecific binding. Furthermore, protease inhibitors such as PMSF, leupeptin, E-64 (made by Peptide Institute), and pepstatin can also be added to suppress protease-induced degradation of CD72 and CD100. A set amount (5000-500,000 cpm) of labeled CD100 is added to 0.01-10 mL of said receptor solution. 10^{-4} - 10^{-1} μM of the test compound is made to be present simultaneously. A test tube with an excess of unlabeled CD100 or salt thereof is also used to investigate nonspecific binding (NSB). The reaction is performed for from 20 minutes to 24 hours, preferably 30 minutes to 3 hours, at from 0 to

50°C, preferably 4 to 37°C. After the reaction, the solution is filtered by glass fiber filter paper or the like and washed with an appropriate amount of the same buffer. The radioactivity remaining in the glass fiber filter paper is measured by liquid scintillation counter or γ-counter. Test compounds with specific binding (B-NSB) of, for example, no more than 50% can be selected as candidate compounds with antagonistic inhibition potential when the count (B_0-NSB) obtained by subtracting the nonspecific binding (NSB) from the count with no antagonist present (B_0) is taken as 100%.

BIAcore (made by Amersham Pharmacia Biotech) can also be used to measure the binding of CD72 or salt thereof and CD100 or salt thereof. In this method, CD100 or salt thereof or derivative thereof is immobilized on the sensor chip by amino coupling according to the instructions included with the equipment. A buffer such as phosphate buffer or Tris buffer that contains CD72 or salt thereof or membrane fraction that contains CD72 or salt thereof purified from cells that contain CD72 or salt thereof or transformants that contain DNA encoding CD72 or that contains purified CD72 or salt thereof or membrane fraction that contains CD72 or salt thereof and test compound is passed over the sensor chip at a flow rate of 2-20 μL/min. Compounds that change the binding property between CD72 or salts thereof and CD100 or salts thereof can be screened by observing how the presence of the test compound affects the changes in surface plasmon resonance produced by binding of CD100 or salt thereof and CD72 or salt thereof on the sensor chip. This method is also capable of similar measurement when CD72 or salt thereof is immobilized on the sensor chip and a buffer such as phosphate buffer or Tris buffer that contains CD100 or salt thereof or CD100 or salt thereof and test compound is passed over the sensor chip. Examples of the test compounds are the same as above.

To implement the aforementioned methods (4) and (5) to screen compounds that change the binding property between CD100 or salts thereof and CD72 or salts thereof,

the immune reaction-enhancing activity mediated by CD72 or salt thereof [e.g., antibody production of antigen-specific IgG; T cell reactivity such as antibody production to TD (T cell-dependent) antigen, T cell growth capacity, IL-4 production, and interferon gamma production; suppression or enhancement of dendritic cell reactivity such as IL-12 production, etc.] can be measured by publicly known methods or using commercial measurement kits.

Specifically, cells that contain CD72 or salt thereof are first cultured in multiwell plates or the like. Prior to conducting screening, the medium is exchanged for fresh medium or a suitable buffer that is nontoxic to the cells. After adding the test compound and incubating for a set length of time, the cells are extracted or the supernatant recovered and the products generated assayed by the respective methods. When degrading enzymes present in the cells make it difficult to study production of compounds that employ immune reaction-enhancing activity as the indicator (e.g., antigen-specific IgG level, interferon gamma, interleukin 12), assay may be performed after adding inhibitors to said degrading enzymes.

Appropriate cells expressing CD72 or salt thereof are used in screening by measuring of the immune reaction-enhancing activity such as antibody production. B cells or the aforementioned recombinant CD72-expressing cell lines are preferred as cells expressing CD72 or salts thereof. It does not matter whether the CD72-expressing cells that are transformants are a stable expression line or a transient expression line. The types of animal cells used are also the same as above.

Examples of test compounds include peptides, proteins, nonpeptide compounds, synthetic compounds, fermentation products, cell extracts, plant extracts, animal tissue extracts, etc.

The following assay system will be used to more specifically explain the above ligand-receptor assay system.

(1) Stimulation of receptor-expressing cells by a receptor agonist causes intracellular class switch and accelerates the production and secretion of all classes of antibodies (IgG, IgA, IgD, IgE) other than IgM. The antibody production-promoting activity of the receptor agonist can be measured directly or indirectly using labeled Ig antibodies by ELISA measurement of the antibody levels produced and secreted. The antibody production-promoting effect of CD100 on cells expressing CD72 can be measured by utilizing this reaction. Specifically, this is done by or in accordance with the method of example 2 discussed below. There, compounds that change the binding property between CD100 or salts thereof and CD72 or salts thereof can be screened by observing changes in antibody production-promoting activity when CD100 or salt thereof or CD100 or salt thereof and test compound are added in comparison to when CD100 or salt thereof is administered alone. Compounds that suppress the antibody production-promoting effect of CD100 on cells expressing CD72 can be selected as candidates with antagonist inhibition potential. On the other hand, agonists can also be screened by administering the test compound alone and observing the antibody production-promoting effect on the cells expressing CD72.

An example of the screening method will be discussed specifically below. 1×10^5 cells/well of spleen resting B cells, prepared by the method described below in example 2, are immobilized by p-formaldehyde together with anti-CD40 monoclonal antibody and 100 units/mL of IL-4 and cultured for approximately 7 days in flat-bottomed 96-well microtiter plates in the presence of normal CHO cells expressing CD100 (2×10^4 cells/well). The IgM or IgG1 immunoglobulin production is measured by ELISA. Specifically, the culture broth or the control IgM or IgG diluted using 0.1M carbonate buffer (pH 9.6) is injected in 100 μL aliquots into each well of an EIA 96-well immunoplate (MaxiSorp: Nunc) and adhered by standing overnight at approximately 4°C. After washing each well

with buffer A (0.02M phosphate buffer, pH 7.0, containing 0.15M NaCl), 100 μL of enzyme-labeled anti-IgM, IgG, IgA, IgD, IgE antibody solution diluted by buffer B (0.02M phosphate buffer, pH 7.0, containing 0.1% BSA and 0.15M NaCl) is added and reacted for another approximately 2 hours at 25°C. Each well is then washed with buffer A, 100 μL of alkali phosphatase substrate solution (1 mg/mL phosphatase substrate (Sigma), 100 mM Tris (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$) added and reacted for 30 minutes at 25°C. The 405 nm absorbance is measured using an automatic microplate colorimeter. Taking the absorbance of the experiment with only CD100 or salt thereof added as 100% and the absorbance of the experiment with no CD100 or salt thereof added as 0%, the effect of the test compound on the promotion of antibody production by CD100 or salt thereof is calculated. Test compounds with antibody production-promoting activity, for example, of no more than 50% can be selected as candidates with antagonistic inhibition potential.

A screening kit for compounds or salts thereof that change the binding property between CD100 or salt thereof and CD72 or salt thereof is one that contains CD72 or salt thereof, cells that contain CD72 or salt thereof or membrane fraction of cells that contain CD72 or salt thereof, and CD100 or salt thereof.

The following can be given as examples of screening kits of the present invention.

1. Screening reagents

(1) Buffer for measurement and buffer for washing

Hanks' Balanced Salt Solution (made by Gibco) with 0.05% bovine serum albumin (made by Sigma) added.

This may be filtered sterilized by a filter with a pore diameter of 0.45 μm and stored at 4°C or may be prepared at the time of use.

(2) CD72 standard

CHO cells expressing CD72 or salt thereof subcultured at 5×10^5 cells/well in 12-well plates and cultured for 2 days at 37°C in 5% CO_2 and 95% air.

(3) Labeled ligand

CD100 or salt thereof labeled by [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc.

This is dissolved in a suitable solvent or buffer, stored at 4°C or -20°C, and diluted to 1 μM by buffer for measurement at the time of use.

(4) Ligand labeling solution

CD100 or salt thereof is dissolved to make 1 mM in PBS that contains 0.1% bovine serum albumin (made by Sigma) and stored at -20°C.

2. Measurement method

(1) After washing cells that have been made to express CD72 or salt thereof cultured in 12-well tissue culture plates twice by 1 mL of buffer for measurement, 490 μL of buffer for measurement is added to each well.

(2) After adding 5 μL of 10^{-3}-10^{-10} M test compound solution, 5 μL of labeled CD100 or salt thereof is added and reacted for 1 hour at room temperature. 5 μL of 10^{-3} M ligand is added instead of test compound to investigate the nonspecific binding.

(3) The reaction solution is removed. The plates are washed three times by 1 mL of buffer for washing. The labeled ligand bonded to the cells is dissolved by 0.2N NaOH-1% SDS and mixed with 4 mL of liquid scintillator A (made by Wako Junyaku).

(4) The radioactivity is measured using a liquid scintillation counter (made by Beckman) and the percent maximum binding (PMB) determined by the following formula.

[Formula 1]

$$PMB=(B-NSB)/(B_0-NSB) \times 100$$

PMB: percent maximum binding

B: a value with specimen added

NSB: nonspecific binding

B_0: maximum binding

The compounds or salts thereof obtained using the screening method or screening kit of the present

invention are compounds that change the binding property between CD100 or salts thereof and CD72 or salts thereof (inhibit or promote binding). Specifically, they are compounds or salts that have an immune reaction-promoting effect such as antibody production mediated by CD72 or salt thereof (so-called CD72 agonists) or compounds that do not have said immune reaction-promoting activity (so-called CD72 antagonists). Examples of said compounds include peptides, proteins, nonpeptide compounds, synthetic compounds, and fermentation products. These compounds may be novel compounds or known compounds.

Concrete methods of evaluating whether a compound is a CD72 agonist or antagonist as above include (i) and (ii) below.

(i) After performing binding assay by a screening method of (1)-(3) above and obtaining a compound that changes the binding property between CD100 or salt thereof and CD72 or salt thereof (particularly one that inhibits binding), one measures whether or not said compound has the aforementioned immune reaction-promoting activity such as CD72-mediated antibody production. Compounds or salts thereof that have immune reaction-promoting activity are CD72 agonists and compounds or salts thereof that do not have said activity are CD72 antagonists.

(ii) (a) The test compound is brought into contact with cells that contain CD72 or salt thereof and the aforementioned immune reaction-promoting activity such as antibody production mediated by CD72 or salt thereof is measured. Compounds or salts thereof with immune reaction-promoting activity are CD72 agonists.

(b) The immune reaction-promoting activity such as antibody production mediated by CD72 or salt thereof is measured and compared when a compound that activates CD72 or salt thereof (e.g., CD100 or CD72 agonist, etc.) is brought into contact with cells that contain CD72 or salt thereof and when a compound that activates CD72 or salt thereof and test compound are brought into contact with cells that contain CD72 or salt thereof. Compounds

or salts thereof capable of decreasing the immune reaction-promoting activity of compounds that activate CD72 or salts thereof are CD72 antagonists.

Said CD72 agonists are useful as safe, low-toxicity drugs in the same way as CD100 or salts thereof because they have similar physiological activity to CD100 and salts thereof on CD72 and salts thereof.

Conversely, CD72 antagonists are useful as safe, low-toxicity drugs that suppress said receptor activity because they are capable of suppressing the physiological activity of CD100 and salts thereof on CD72 and salts thereof.

Moreover, CD72 and salts thereof are useful as safe, low-toxicity drugs because they are capable of suppressing the physiological activity of CD100 and salts thereof in the same way as CD72 antagonists.

CD100 and salts thereof can be used as antibody production inducers, immunopotentiators and the like because they induce class switch and promote antibody production. Therefore, CD72 agonists, among the compounds obtained using the aforementioned screening method or screening kit, can be used in the prevention and treatment of viral infections and diseases (such as colds, influenza, AIDS, hepatitis, herpes, measles, varicella, foot-and-mouth disease, herpes zoster, erythema infectiosum, rubella, exanthema subitum, viral conjunctivitis, viral meningitis, viral pneumonia, viral encephalitis, Lassa fever, Ebola hemorrhagic fever, Marburg disease, Congo hemorrhagic fever, yellow fever, dengue fever, rabies, adult T cell leukemia (ATL), rotavirus infection, polio, mumps, etc.), infections and diseases caused by bacteria or fungi (such as bacterial food poisoning, bacterial diarrhea, tuberculosis, Hansen's disease, dysentery, typhoid fever, cholera, paratyphus, plague, tetanus, tularemia, brucellosis, anthrax, septicemia, bacterial pneumonia, dermatomycosis, etc.), and cancer (oral cavity cancer, pharyngeal cancer, lip cancer, tongue cancer, gingival cancer, nasopharyngeal cancer, esophageal cancer, stomach cancer,

small intestinal cancer, large intestinal cancer including colon cancer, liver cancer, gallbladder cancer, pancreatic cancer, nasal cavity cancer, lung cancer, bone cancer, soft tissue cancer, skin cancer, melanoma, breast cancer, uterine cancer, ovarian cancer, prostate cancer, testicular cancer, cancer of the penis, urinary bladder cancer, kidney cancer, brain tumor, thyroid cancer, lymphoma, leukemia, etc.), etc. CD72 antagonists (or CD72 or salts thereof) can be used in the prevention and treatment of diseases caused by abnormal antibody production or excessive antibody production (such as atopic asthma, allergic rhinitis, atopic dermatitis, allergic bronchitis, pulmonary aspergillosis, parasitic disease, Kimura's disease, hyper-IgE syndrome, Wiskott-Aldrich syndrome, thymic aplasia, Hodgkin's disease, liver cirrhosis, acute hepatitis, chronic rheumatoid arthritis, insulin-dependent diabetes, systemic erythematosus, scleroderma, infertility, endometriosis, autoimmune thyroid disease myasthenia gravis [sic], Hashimoto's disease, Basedow's disease, pernicious anemia, Addison's disease, male infertility, multiple sclerosis, Goodpasture syndrome, pemphigus, pemphigoid, myasthenia gravis, ophthalmia of the lens, sympathetic ophthalmia, autoimmune hemolytic anemia, idiopathic thrombocytopenia, autoimmune leukopenia, Felty syndrome, autoimmune lymphocytopenia, ulcerative colitis, Sjogren's syndrome, systemic autoimmune disease, primary biliary liver cirrhosis, lupoid hepatitis, etc.).

Pharmacologically acceptable salts, for example, are used as the salts of compounds obtained using the aforementioned screening method or screening kit. Examples include salts of inorganic bases, salts of organic bases, salts of inorganic acids, salts of organic acids, and salts of basic and acidic amino acids.

Examples of salts of inorganic bases include alkali metal salts such as sodium and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, aluminum salts, and ammonium salts.

Examples of suitable salts of organic bases include salts of triethylamine, trimethylamine, pyridine, picoline, 2,6-lutidine, ethanolamine, diethanolamine, triethanolamine, cyclohexylamine, dicyclohexylamine, and N,N'-dibenzylethylenediamine.

Examples of suitable salts of inorganic acids include salts of hydrochloric acid, hydrobromic acid, sulfuric acid, and phosphoric acid.

Examples of suitable salts of organic acids include salts of formic acid, acetic acid, propionic acid, fumaric acid, oxalic acid, tartaric acid, maleic acid, citric acid, succinic acid, malic acid, methanesulfonic acid, benzenesulfonic acid, and benzoic acid.

Examples of suitable salts of basic amino acids include salts of arginine, lysine, and ornithine. Examples of suitable salts of acidic amino acids includes salts of aspartic acid and glutamic acid.

Ordinary means can be used when compounds or salts thereof obtained using the screening method or screening kit of the present invention are used as drugs. For example, they can be used orally as tablets, sugar- or enteric-coated if necessary, capsules, elixirs, microcapsules, etc., or nonorally as injections such as suspensions or sterile solutions of water or other pharmacologically acceptable solutions. For example, drugs can be manufactured by mixing said compounds or salts thereof together with physiological carriers, flavorings, excipients, vehicles, preservatives, stabilizers, binders, etc., in common unit-dose form required for drug manufacture. The amount of active ingredient in these preparations is such that an appropriate volume is obtained within the designated range.

Binders such as gelatin, corn starch, tragacanth gum, and gum arabic, excipients such as crystalline cellulose, swelling agents such as corn starch, gelatin and alginic acid, lubricants such as magnesium stearate, sweeteners such as sucrose, lactose, and saccharine, and flavorings such as peppermint, wintergreen oil, and cherry, etc.,

are used as additives that can be mixed with tablets and capsules, etc. When the unit form of the preparation is a capsule, it can also contain a liquid carrier such as an oil in addition to the aforementioned types of materials. Sterile compositions for injection can be formulated in accordance with ordinary drug preparation by dissolution or suspension of the active substance and a naturally-produced vegetable oil such as sesame oil or coconut oil in the vehicle such as water for injection.

Examples of aqueous solutions for injection include physiological saline and isotonic solutions that contain glucose and other auxiliaries (e.g., D-sorbitol, D-mannitol, sodium chloride, etc.). Alcohols (e.g., ethanol), polyalcohols((e.g., propylene glycol, polyethylene glycol), nonionic surfactants (e.g., Polysorbate 80™, HCO-50), etc., may be used in combination as suitable dissolution auxiliaries. Examples of oily solutions include sesame oil and soybean oil. Benzyl benzoate, benzyl alcohol, etc. may be used in combination as dissolution auxiliaries.

Buffers (e.g., phosphate buffer, sodium acetate buffer), analgesics (e.g., benzalkonium chloride, procaine hydrochloride, etc.), stabilizers (e.g., human serum albumin, polyethylene glycol, etc.), preservatives (e.g., benzyl alcohol, phenol, etc.), antioxidants, etc., may also be combined. The prepared injectable solution is usually packaged in appropriate ampules.

The preparations obtained in this way can be administered, for example, to humans and mammals (e.g., mice, rats, guinea pigs, rabbits, sheep, pigs, cows, cats, dogs, monkeys, chimpanzees, etc.) because they are safe and low in toxicity.

The dose of the compounds or salts thereof obtained using the screening method or screening kit of the present invention varies depending on the symptoms, etc. However, in oral administration to adults (body weight 60 kg), it is generally from approximately 0.1 to 1000 mg, preferably approximately 1.0 to 300 mg, more preferably approximately 3.0 to 50 mg, per day. In nonoral

administration, the one-time dose also differs depending on the reason for administration, subject organ, symptoms, administration method, etc. However, for example, it is usually appropriate in administration to an adult (body weight 60 kg) in the form of an injection to administer from approximately 0.01 to 30 mg, preferably approximately 0.1 to 20 mg, more preferably approximately 0.1 to 10 mg, per day by intravenous injection.

Other animals can also be administered doses calculated per 60 kg.

When CD72 or salts thereof are used as drugs, they can be made into preparations and used in the same way as when compounds or salts thereof obtained by the screening method or screening kit of the present invention as described above are used as drugs.

[Nonhuman animals with knockout CD100 gene]

Nonhuman animals with knockout CD100 gene (termed CD100 gene nonexpressing nonhuman animals below) can be produced using nonhuman animal ES cells with an inactivated CD100 gene sequence.

The term nonhuman animal ES cells with inactivated CD100 gene sequence means inactivated nonhuman animal ES cells that essentially do not possess the ability of the gene to express CD100 (sometimes referred to hereinafter as knockout gene) by suppressing the expression capacity of the gene by applying artificial modifications to the CD100 gene of the nonhuman animal ES cells or causing essential loss of the activity of CD100 coded by said gene.

The nonhuman animals may be any animals as long as they are animals other than humans that possess the CD100 gene. Nonhuman mammals are preferred. Cows, pigs, sheep, goats, rabbits, dogs, cats, guinea pigs, hamsters, mice, rats, etc., are used as nonhuman mammals. Among the nonhuman mammals, rodents, especially mice (e.g., C57BL/6 and DBA2 lines as pure lines and B6C3F1, BDF1, B6D2F1, BALB/c, and ICR lines as hybrid lines) and rats (e.g., Wistar, SD, etc.), are especially preferred from the

standpoint of preparation of pathology models in animals for their relatively short individual development and lifecycles and ease of breeding.

The CD100 gene may be a genome-derived CD100 gene isolated and extracted from an animal or CD100 cDNA cloned using genetic engineering techniques.

Specifically, genes with the base sequence shown by SEQ ID NO: 2, for example, are used as genes of mouse CD100 protein with the amino acid sequence by SEQ ID NO: 1. Genes with the base sequence shown by SEQ ID NO: 4, for example, are used as genes of human CD100 protein with the amino acid sequence shown by SEQ ID NO: 3. These genes can be obtained by the methods described above.

Artificial modificatioin of the CD100 gene can be effected by deletion of some or all of said gene sequence or by insertion or substitution or other genes by genetic engineering techniques. These modifications make it possible to create CD100 knock-out genes, for example, by shifting the codon reading frame or disrupting the function of the promoter or exon.

Concrete examples of nonhuman animal embryonic stem cells with an inactivated CD100 gene sequence (abbreviated hereinafter as CD100 gene inactivated ES cells or knock-out ES cells) include disruption of exon function by insertion of a drug resistance gene (e.g., neomycin resistance gene, hygromycin resistance gene, etc., preferably omycin resistance gene, etc.) or reporter gene (e.g., lacZ (β-galactosidase gene), cat (chloramphenicol acetyl transferase gene), etc. preferably lacZ, etc.) or creation of a DNA strand with the DNA sequence constructed so as to result in disruption of the gene (abbreviated hereinafter as targeting vector) by making complete messenger RNA synthesis impossible by inserting a DNA sequence (e.g., polyA addition signal, etc.) to end gene transcription in the intron region between exons. When exon function is disrupted by inserting a reporter gene, said reporter gene is preferably inserted so that it is expressed under the control of the CD100 promoter.

A DNA strand with a DNA sequence constructed so as to disrupt the gene can be obtained, for example, by selecting knockout ES cells by southern hybridization analysis of ES cells obtained by introduction into the chromosomes of said animal by homologous recombination using a DNA sequence on or near the CD100 gene as the probe or by analysis by PCR using the DNA sequence on the targeting vector and a nearby DNA sequence other than the CD100 gene used in production of the targeting vector as the primer.

The original ES cells in which the CD100 gene has been inactivated by homologous recombination, etc. may be already established ones as described above or may be newly established by the method of Evans and Kaufma. For example, ES cells of line 129 are generally used today in the case of mouse ES cells. However, since their immunological background has not been clarified, it may be preferable to use cells established using BDF1 mice (F1 of C57BL/6 and DBA/2) improved by crossbreeding C57BL/6 mice and a small number of eggs collected from C57BL/6 with DBA/2, for example, in order to acquire ES cells with an immunologically clear genetic background in pure strains rather than the above. Since BDF1 mice have the background of C57BL/6 mice in addition to advantages such as a large number of eggs collected and durable eggs, ES cells obtained using them can be used advantageously as it is possible to substitute their genetic background in C57BL/6 mice by backcrossing with C57BL/6 mice when producing pathology model mice.

Blastocysts 3.5 days after fertilization are generally used for establishing ES cells. However, many early embryos can also be acquired efficiently by harvesting eight-cell embryos and culturing into blastocysts.

Although ES cells of both males and females may be used, ES cells of males are usually better for making reproduction system chimeras. It is preferable to distinguish males and females as early as possible to eliminate time-consuming, complicated culture.

One example of a means of distinguishing male and female ES cells is amplification and detection of genes in the sex determination region on the Y chromosome by PCR. Since use of this method consumes a number of ES cells equivalent to one colony (approximately 50) as opposed to the approximately 10^6 cells needed for karyotype analysis in the past, it makes it possible to base primary selection of ES cells on discrimination of males and females in the early stage of culture and greatly reduces the time and effort involved in the early stage of culture by making it possible to select the male cells early.

The secondary selection can be done, for example, by verification of chromosome number by G-banding. Although the chromosome number of the ES cells obtained is preferably 100% of the usual number, it is preferable to again clone to normal cells (e.g., cells with a chromosome number of 2n = 40 in mice) after knockout of the gene on the ES cell when difficulty is encountered in relationship to the physical procedure, etc. in establishing the line.

The growth capacity of the embryonic stem cell line obtained in this way is usually very good. However, careful subculture is necessary since individual developmental capacities tend to be lost. For example, methods include culturing by a method such as culture at approximately 37°C on appropriate feeder cells such as STO fibroblasts in the presence of LIF (1-10,000 U/mL) in a carbon dioxide incubator (preferably, 5% carbon dioxide and 95% air or 5% oxygen, 5% carbon dioxide, and 90% air) and placement on fresh, unused fetal cells after producing single cells, for example, by trypsin/EDTA solution (usually 0.001-0.5% trypsin-0.1-5 mM EDTA, preferably approximately 0.1% trypsin/1 mM EDTA) treatment during subculture. Such subculture is usually performed every 1-3 days. However, it is preferable to examine the cells at this time and discard any cultured cells that appear morphologically abnormal.

ES cells can be differentiated into various types of cells such as parietal muscle, visceral muscle, and cardiac muscle by monolayer culture to high density under appropriate conditions or suspended culture until cell masses form [M. J. Evans and M. H. Kaufman, Nature, Vol. 292, p. 154, 1981; G. R. Martin, Proc. Natl. Acad. Sci. USA, Vol. 78, p. 7634, 1981; T. C. Doeischman et al., Journal of Embryology and Experimental Morphology, Vol. 87, p. 27, 1985]. The CD100 gene nonexpressing cells obtained by differentiation of the ES cells are useful in cytological studies of CD100 in the intron.

The term transgenic nonhuman animals with an inactivated CD100 gene sequence (sometimes referred to hereinafter as gene nonexpressing nonhuman animals) means nonhuman animals prepared by genetic engineering using cells derived from the aforementioned nonhuman animal embryonic stem cells with an inactivated CD100 gene sequence, e.g., nonhuman animals with said inactivated CD100 gene sequence inserted into reproductive and somatic cells at an early stage in embryonic development.

The same ones as above are used as said nonhuman animals.

The CD100 gene can be knockout by introducing the aforementioned targeting vector into mouse embryonic stem cells or mouse egg cells and inserting the inactivated CD100 gene sequence of the targeting vector in place of the CD100 gene on the chromosomes of the mouse embryonic stem cells or mouse egg cells by genetic homologous recombination.

Cells with a knockout CD100 gene can be determined by southern hybridization analysis using a DNA sequence on or near the CD100 gene as the probe or by analysis by PCR using as the primer the DNA sequence on the targeting vector and a DNA sequence in a nearby region other than the mouse CD100 gene used in the targeting vector.

When nonhuman animal embryonic stem cells have been used, cell lines with the CD100 gene inactivated are cloned by homologous recombination, these cells injected into nonhuman animal embryos or blastocysts at a suitable

point in early embryonic development, e.g., the eight-cell stage, and transplanted to the uteri of said nonhuman animals made falsely pregnant by the prepared chimera embryos.

The animals prepared are chimera animals constructed from both cells with a normal CD100 gene locus and cells with an artificially modified CD100 gene locus.

When some of the reproductive cells of said chimera animals have the modified CD100 gene locus, individuals in which all tissues are constructed from cells with the artificially modified CD100 gene locus are obtained, for example, by selection based on coat color from among a group of individuals obtained by mating these chimera individuals and normal individuals. The individuals obtained in this way are usually CD100 hetero-type insufficient expressing individuals. CD100 homo-type insufficient expressing individuals can be obtained from their progeny by mating the CD100 hetero-type insufficient expressing individuals to each other.

When egg cells are used, transgenic nonhuman animals with the targeting vector introduced into the chromosome can be obtained, for example, by injecting genetic solution into the egg cell nuclei by microinjection. Those with mutation at the CD100 gene locus are selected by homologous recombination in comparison [sic] to these transgenic nonhuman animals.

The CD100 gene nonexpressing nonhuman animals can be distinguished from normal animals by indirectly comparing the expression levels by measuring the mRNA of said animals by publicly known methods.

Individuals with a knockout CD100 gene obtained in this way can be bred through generations under normal rearing conditions after confirming knockout of said gene in the individual animals obtained by breeding as well.

Moreover, a reproduction system can be acquired and maintained by ordinary methods. Specifically, homozygote animals with said inactivated gene sequence in both homologous chromosomes can be acquired by mating male and female animals that possess said inactivated gene

sequence. The homozygote animals obtained can be obtained efficiently by breeding to the maternal parents so that there is one normal individual and multiple homozygotes. Homozygote and heterozygote animals with said inactivated gene sequence can be bred through the generations by mating heterozygote male and female animals. The progeny of animals with said inactivated gene sequence obtained in this way also include insufficient CD100-expressing nonhuman animals.

Thus, nonhuman animal embryoic stem cells with the CD100 gene inactivated are extremely useful for making CD100 gene nonexpressing nonhuman animals.

The CD100 gene nonexpressing nonhuman animals prepared as described above are nonhuman animals with (1) decreased antibody production capacity to TD (T cell-dependent) antigen (example 6 discussed below), (2) loss of T cell reactivity such as T cell growth capacity, IL-4 production capacity, and INF-γ production capacity (example 7 discussed below), and (3) and loss of reactivity of dendritic cells such as IL-12 production capacity (example 10 discussed below). Since they can serve as models of diseases caused by CD100 deletion, e.g., diseases caused by inactivation of the bioactivities of CD100 due to loss of the various bioactivities induced by CD100, e.g., infections and diseases (such as colds, influenza, AIDS, hepatitis, herpes, measles, varicella, foot-and-mouth disease, herpes zoster, erythema infectiosum, rubella, exanthema subitum, viral conjunctivitis, viral meningitis, viral pneumonia, viral encephalitis, Lassa fever, Ebola hemorrhagic fever, Marburg disease, Congo hemorrhagic fever, yellow fever, dengue fever, rabies, adult T cell leukemia (ATL), rotavirus infection, polio, mumps, etc.), infections and diseases caused by bacteria or fungi (such as bacterial food poisoning, bacterial diarrhea, tuberculosis, Hansen's disease, dysentery, typhoid fever, cholera, paratyphus, plague, tetanus, tularemia, brucellosis, anthrax, septicemia, bacterial pneumonia, dermatomycosis, etc.), and cancer (oral cavity cancer,

pharyngeal cancer, lip cancer, tongue cancer, gingival cancer, nasopharyngeal cancer, esophageal cancer, stomach cancer, small intestinal cancer, large intestinal cancer including colon cancer, liver cancer, gallbladder cancer, pancreatic cancer, nasal cavity cancer, lung cancer, bone cancer, soft tissue cancer, skin cancer, melanoma, breast cancer, uterine cancer, ovarian cancer, prostate cancer, testicular cancer, cancer of the penis, urinary bladder cancer, kidney cancer, brain tumor, thyroid cancer, lymphoma, leukemia, etc.), they are useful in explaining the causes and studying methods of treating these conditions.

In other words, CD100 gene nonexpressing nonanimals [sic] can be used to screen drugs for the prevention and treatment of said diseases.

The same ones as above can be given as examples of the CD100 gene nonexpressing nonhuman animals used in the screening method of the present invention.

Examples of test compounds include peptides, proteins, nonpeptide compounds, synthetic compounds, fermentation products, cell extracts, plant extracts, and animal tissue extracts. These compounds may be either novel compounds or known compounds.

Specifically, the preventative and therapeutic effects of the test compounds can be tested by treating CD100 gene nonexpressing nonhuman animals with the test compound and using changes in the organs, tissues, diseases symptoms, etc., of said animals as indicators in comparison to untreated control animals.

Oral administration, intravenous injection, etc. can be used to treat the test animals by the test compounds. The method can be selected as is appropriate to the symptoms of the test animals, properties of the test compound, etc. The dose of the test compound can also be selected as is appropriate to the administration method, properties of the test compound, etc.

For example, when screening drugs for the prevention and treatment of cancer, the test compound is administered over time intraperitoneally, subcutaneously,

intravenously, etc., to the CD100 gene nonexpressing nonhuman animals, for example, following administration of keyhole limpet hemocyanine. Screening is then performed by measuring the interferon gamma or IL-12 levels in the blood. Furthermore, screening is also possible by measuring the tumor volume and duration of survival by transplanting tumors intraperitoneally, subcutaneously, or intravenously and administering the test compound over time intraperitoneally, subcutaneously, intravenously, etc.

The preventative and therapeutic drugs obtained by the screening method of the present invention using nonhuman animals with a knockout CD100 gene are compounds selected from among the aforementioned test compounds. Since they have preventative and therapeutic effects in diseases caused by CD100 deletion, they are useful as drugs such as safe, low-toxicity, therapeutic and preventative drugs for diseases triggered by CD100 deletion. Compounds derived from said compounds can also be used in the same way.

The compounds obtained by said screening method may form salts. Physiologically acceptable acid addition salts are especially preferred as salts of said compounds. For examples, salts of inorganic acids (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid) or salts of organic acids (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid) are used as such salts.

The screening method of the present invention that uses nonhuman animals with a knockout CD100 gene may be combined with the aforementioned screening method for compounds or salts thereof that change the binding property between CD100 and CD72 (ligand-receptor assay system). Specifically, the present invention proposes a screening method for compounds (particularly compounds that promote binding of CD100 and CD72 and compounds that bind to CD72 in place of CD100) or salts thereof that

change the binding property between CD100 or salts thereof and receptors thereof characterized by using nonhuman animals with a knockout CD100 gene.

Here, after selecting compounds that change the binding property between CD100 and CD72 as candidate compounds using the ligand-receptor assay system as the primary screening, the preventative and therapeutic effects of said candidate compounds may be tested in a secondary screening system using nonhuman animals with a knockout CD100 gene. Or, candidate compounds are selected by primary screening using a screening system that employs nonhuman animals with the CD100 gene knockout, then compounds that change the binding property between CD100 and CD72 obtained using the ligand-receptor assay system for secondary screening may be selected as candidate compounds for the preventative and therapeutic drugs of the present invention.

The compounds or salts thereof obtained by the screening method of the present invention using nonhuman animals with the CD100 gene knockout can be used as the aforementioned therapeutic and preventative drugs by ordinary means. For example, they can be used orally as tablets, sugar-coated if necessary, capsules, elixirs, microcapsules, etc., or nonorally in the form of injections such as suspensions or sterile solutions together with water or another pharmacologically acceptable solution. For example, these can be manufactured by mixing said compounds or salts thereof together with physiological carriers, flavorings, excipients, vehicles, preservatives, stabilizers, binders, etc., in the unit dose form generally required in drug manufacture. The amount of active ingredient in these preparations is set so as to obtain an appropriate volume within the indicated range.

Binders such as gelatin, corn starch, tragacanth gum, and gum arabic, excipients such as crystalline cellulose, swelling agents such as corn starch, gelatin and alginic acid, lubricants such as magnesium stearate, sweeteners such as sucrose, lactose, and saccharine, and flavorings

such as peppermint, wintergreen oil, and cherry, etc., are used as additives that can be mixed with tablets and capsules, etc. When the unit form of the preparation is a capsule, it can also contain a liquid carrier such as an oil in addition to the aforementioned types of materials. Sterile compositions for injection can be formulated in accordance with ordinary drug preparation by dissolution or suspension of the active substance and a naturally-produced vegetable oil such as sesame oil or coconut oil in the vehicle such as water for injection.

Examples of aqueous solutions for injection include physiological saline and isotonic solutions that contain glucose and other auxiliaries (e.g., D-sorbitol, D-mannitol, sodium chloride, etc.). Alcohols (e.g., ethanol), polyalcohols((e.g., propylene glycol, polyethylene glycol), nonionic surfactants (e.g., Polysorbate 80™, HCO-50), etc., may be used in combination as suitable dissolution auxiliaries. Examples of oily solutions include sesame oil and soybean oil. Benzyl benzoate, benzyl alcohol, etc. may be used in combination as dissolution auxiliaries. Buffers (e.g., phosphate buffer, sodium acetate buffer), analgesics (e.g., benzalkonium chloride, procaine hydrochloride, etc.), stabilizers (e.g., human serum albumin, polyethylene glycol, etc.), preservatives (e.g., benzyl alcohol, phenol, etc.), antioxidants, etc., may also be combined. The prepared injectable solution is usually packaged in appropriate ampules.

Since the preparations obtained in this way are safe and low in toxicity, they can be administered, for example, to humans and warm-blooded animals (e.g., mice, rats, rabbits, sheep, pigs, cows, horses, chickens, cats, dogs, monkeys, chimpanzees, etc.).

The dose of the compounds or salts thereof varies depending on the symptoms, etc. In oral administration, it is generally approximately 0.1-100 mg, preferably approximately 1.0-50 mg, more preferably approximately 1.0-20 mg, per day for adults (body weight 60 kg). In nonoral administration, the one-time dose also differs

depending on the administration subject, subject organ, symptoms, method of administration, etc. However, in the case of an injection, for example, it is appropriate to administer approximately 0.01-30 mg, preferably approximately 0.1-20 mg, more preferably approximately 0.1-10 mg, by intravenous injection per day for ordinary adults (as 60 kg). Other animals can also be administered doses calculated per 60 kg.

The present invention also proposes a screening method for compounds or salts thereof that promote or inhibit CD100 promoter activity characterized by detecting expression of the reporter gene by administering test compounds to CD100 gene nonexpressing nonhuman animals.

Those that have an inactivated CD100 gene sequence due to introduction of a reporter gene and are capable of expressing said reporter gene under the control of the CD100 promoter are used among the aforementioned CD100 gene nonexpressing nonhuman animals as the CD100 gene nonexpressing nonhuman animals employed in the screening method of the present invention.

The same ones as above can be given as examples of test compounds.

The same ones as above are used as the reporter gene. It is especially preferred to use a β-galactosidase gene (lacZ).

Since the reporter gene is present under the control of the CD100 promoter in animals expressing CD100 in which a structural gene of CD100 has been substituted by the reporter gene, the CD100 promoter activity can be detected by tracing expression of the compound coded by the reporter gene.

For example, when a portion of the gene region encoding CD100 is substituted by a β-galactosidase gene (lacZ) derived from *E. coli*, β-galactosidase is expressed instead of CD100 in tissues that previously expressed CD100. Therefore, the state of expression of CD100 in the animal body can be observed simply, for example, by staining using a reagent that serves as a substrate of β-

galactosidase such as 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal). Specifically, a CD100-deficient mouse or tissue slices thereof are fixed by glutaraldehyde, etc. and washed by Dulbecco's phosphate-buffered physiological saline solution (PBS). After reacting for from approximately 30 minutes to 1 hour near room temperature or 7°C with staining solution that contains X-gal, the β-galactosidase reaction is stopped by washing the tissue specimens with 1 mM EDTA/PBS solution. The coloration may then be examined. The mRNA encoding lacZ may also be detected by the usual method.

Thus, CD100 gene nonexpressing nonhuman animals are extremely useful for the screening of compounds or salts thereof that promote or inhibit and inactivate the CD100 promoter. This can contribute significantly to the explanation of causes and development of drugs for the prevention and treatment of various diseases caused by imperfect CD100 expression.

The compounds or salts thereof obtained using the aforementioned screening method are compounds selected from among the aforementioned test compounds and are compounds that promote or inhibit CD100 promoter activity.

Since compounds or salts thereof that promote CD100 promoter activity accelerate the expression of CD100 and enhance CD100 function, they are useful as drugs such as safe, low-toxicity drugs for the treatment and prevention of various diseases such as infections and diseases caused by viruses (such as colds, influenza, AIDS, hepatitis, herpes, measles, varicella, foot-and-mouth disease, herpes zoster, erythema infectiosum, rubella, exanthema subitum, viral conjunctivitis, viral meningitis, viral pneumonia, viral encephalitis, Lassa fever, Ebola hemorrhagic fever, Marburg disease, Congo hemorrhagic fever, yellow fever, dengue fever, rabies, adult T cell leukemia (ATL), rotavirus infection, polio, mumps, etc.), infections and diseases caused by bacteria or fungi (such as bacterial food poisoning, bacterial diarrhea, tuberculosis, Hansen's disease, dysentery, typhoid fever, cholera, paratyphus, plague, tetanus, tularemia,

brucellosis, anthrax, septicemia, bacterial pneumonia, dermatomycosis, etc.), and cancer (oral cavity cancer, pharyngeal cancer, lip cancer, tongue cancer, gingival cancer, nasopharyngeal cancer, esophageal cancer, stomach cancer, small intestinal cancer, large intestinal cancer including colon cancer, liver cancer, gallbladder cancer, pancreatic cancer, nasal cavity cancer, lung cancer, bone cancer, soft tissue cancer, skin cancer, melanoma, breast cancer, uterine cancer, ovarian cancer, prostate cancer, testicular cancer, cancer of the penis, urinary bladder cancer, kidney cancer, brain tumor, thyroid cancer, lymphoma, leukemia, etc.), etc.

On the other hand, compounds and salts thereof that inhibit CD100 promoter activity can inhibit CD100 expression and inhibit CD100 function. Therefore, they are useful as drugs such as safe, low-toxicity drugs for the treatment and prevention of diseases caused by abnormal antibody production or excessive antibody production (e.g., atopic asthma, allergic rhinitis, atopic dermatitis, allergic bronchitis, pulmonary aspergillosis, parasitic disease, Kimura's disease, hyper-IgE syndrome, Wiskott-Aldrich syndrome, thymic aplasia, Hodgkin's disease, liver cirrhosis, acute hepatitis, chronic rheumatoid arthritis, insulin-dependent diabetes, systemic erythematosus, scleroderma, infertility, endometriosis, autoimmune thyroid disease myasthenia gravis [sic], Hashimoto's disease, Basedow's disease, pernicious anemia, Addison's disease, male infertility, multiple sclerosis, Goodpasture syndrome, pemphigus, pemphigoid, myasthenia gravis, ophthalmia of the lens, sympathetic ophthalmia, autoimmune hemolytic anemia, idiopathic thrombocytopenia, autoimmune leukopenia, Felty syndrome, autoimmune lymphocytopenia, ulcerative colitis, Sjogren's syndrome, systemic autoimmune disease, primary biliary liver cirrhosis, lupoid hepatitis, etc.). When the compounds or salts thereof obtained using the aforementioned screening method are used as therapeutic or preventative drugs as

described above, this can be done in the same way as above.

[CD100 transgenic animals]

Transgenic nonhuman animals that have DNA that incorporates an exogenous CD100 gene or mutated gene thereof (abbreviated hereinafter as CD100 transgenic animals) can be made by transferring the target CD100 gene by the calcium phosphate method, electrical pulse method, lipofection, aggregation, microinjection, particle gun, DEAE-dextran method, etc., to a reproductive cell, including a nonfertilized egg, fertilized egg, sperm, or progenitor cells thereof, preferably at an early stage of embryogenesis (even more preferably at the single-cell or fertilized egg cell stage, generally before the eight-cell stage) during the development of the nonhuman animal. Said CD100 gene transfer techniques transfer the target exogenous CD100 gene to the somatic cells, body organs, tissue cells, etc., and can be used in cell culture and tissue culture, etc. Furthermore, CD100 transgenic animals can also be prepared by fusing these cells with the aforementioned reproductive cells by known cell fusion techniques.

The same ones as above are used as the nonhuman animals.

CD100 genes isolated and extracted from any animals that are not the CD100 gene present in the body of the nonhuman animal and CD100 cDNA cloned by genetic engineering techniques, etc. are used as the exogenous CD100 gene.

Specifically, genes with the aforementioned base sequences shown by SEQ ID NO:.: 2 or SEQ ID NO:.: 4 are used.

Those with a modification (e.g., mutation, etc.) in the base sequence of the original CD100 gene, specifically, CD100 genes with base addition, deletion, substitution of other bases, etc., are used as mutated genes of the CD100 gene (abbreviated hereinafter as modified CD100 genes). These also include abnormal genes

of the CD100 gene (abbreviated hereinafter as abnormal CD100 genes).

Said term abnormal CD100 gene means a CD100 gene that expresses abnormal CD100. For example, a CD100 gene that expresses CD100 that suppresses normal CD100 function is used.

The exogenous CD100 gene may derive from the same or different species as the subject animal.

The DNA that incorporates the exogenous CD100 gene or mutated gene thereof may be any as long as it is DNA that contains the exogenous CD100 gene or mutated gene thereof.

It is generally convenient in transferring the CD100 gene to the subject animal to use a DNA construct with said CD100 gene bonded downstream of the promoter capable of expression in animal cells. For example, when transferring a human CD100 gene, a CD100 transgenic animal with high expression of the CD100 gene can be prepared by microinjection of a DNA construct (e.g., vector, etc.) in which the human CD100 gene has been bonded downstream of various promoters capable of expressing CD100 genes derived from various nonhuman animals that have high CD100 gene homology (e.g., rabbits, dogs, cats, guinea pigs, hamsters, rats, mice, etc.).

E. coli plasmids, *B. subtilis* plasmids, yeast plasmids, bacteriophages such as lambda phage, and animal viruses such as retroviruses such as Moloney's leukemia virus, vaccinia virus, and baculovirus, etc., are used as the CD100 expression vector. It is preferable to use *E. coli* plasmids, *B. subtilis* plasmids, or yeast plasmids among them.

Promoters derived from viruses (e.g., simian virus, cytomegalovirus, Moloney's leukemia virus, JC virus, breast cancer virus, polio virus, etc.) and promoters derived from animals (humans, rabbits, dogs, cats, guinea pigs, hamsters, rats, mice, etc.) and birds (chickens, etc.) such as albumin, insulin II, uroplakin II, elastase, erythropoietin, endothelin, muscle creatinine kinase, glial fibrillary acidic protein, glutathione

S-transferase, platelet growth factor β, keratin K1, K10 and K14, types I and II collagen, cyclic AMP-dependent protein kinase β-I subunit, dystrophin, tartrate-resistant alkaline phosphatase, atrial natriuretic factor, endothelial receptor tyrosine kinase (generally termed Tie2), sodium-potassium adenosine triphosphatase (Na, K-ATPase), neurofilament light chain, metallothioneine I and IIA, metalloproteinase I tissue inhibitor, MHC I antigen (H-2L), H-ras, renin, dopamine β-hydroxylase, thyroid peroxidase (TPO), polypeptide chain growth factor 1α (EF-1α), β-actin, α and β myosin heavy chain, myosin light chain 1 and 2, myelin basic protein, thyroglobulin, Thy-1, immunoglobin, H chain variable reaction (VNP), serum amyloid P component, myoglobin, troponin C, smooth muscle α actin, preproenkephalin A, vasopressin, etc., are used as the aforementioned promoter that regulates CD100 gene expression. It is preferable to use a cytomegalovirus promoter, human polypeptide chain growth factor 1α (EF-1α) promoter, or human or chicken β-actin promoter that permit high expression systemically. An insulin promoter, albumin promoter, immunoglobulin promoter, etc., that allows high expression in specific organs and tissues can also be used.

The aforementioned vector preferably has a sequence that stops the transcription of the target messenger RNA (generally called a terminator) in the CD100 transgenic animal. For example, sequences of various CD100 genes derived from viruses, mammals, and birds can be used. It is preferable to use a simian virus SV40 terminator, etc.

A gene splicing signal, enhancer region such as an immunoglobulin gene, partial eukaryotic cell gene intron, etc., can also be linked 5' upstream of the promoter region, between the promoter region and translation region, or 3' downstream of the translation region in accordance with the goal to induce even higher expression of the target CD100 gene.

The normal CD100 translation region can be acquired using as the raw material all or part of genomic genes from DNA derived from the liver, kidneys, thyroid cells,

or fibroblast cells of various animals (e.g., rabbits, dogs, cats, guinea pigs, hamsters, rats, mice, humans, etc.) and various commercial genome libraries or using complement CD100 gene prepared by a known method from RNA derived from liver, kidney, thyroid cells, or fibroblasts. Exogenous abnormal CD100 genes can also be produced by modifying the normal CD100 translation region obtained from the aforementioned cells or tissues by a point mutation induction technique.

Said translation region can be produced by ordinary genetic engineering techniques that cause linking downstream of the aforementioned promoter and upstream of the transcription end site if desired as a DNA construct that can be expressed in the transgenic animals.

Transfer of the CD100 gene in the fertilized egg cell stage assures its presence in all of the reproductive cells and somatic cells of the subject animal. The presence of the CD100 gene in the reproductive cells of the animal prepared after transfer of the CD100 gene means that all of the reproductive cells and somatic cells in all subsequent generations of the animal prepared possess the CD100 gene. Progeny of this type of animal that has inherited the CD100 gene have the CD100 gene in all of their reproductive cells and somatic cells.

Nonhuman animals to which the exogenous normal CD100 has been transferred can be bred through the generations in an ordinary rearing environment as animals that possess said CD100 gene after confirming by breeding that the CD100 gene is held stably. Transfer of the CD100 gene in the fertilized egg cell stage assures an excess in all of the reproductive cells and somatic cells of the subject animal. The presence of an excess of CD100 gene in the reproductive cells of the animals prepared after CD100 gene transfer means that the progeny of the animals prepared will have an excess of CD100 gene in all of their reproductive cells and somatic cells. The progeny of this type of animal that have inherited the CD100 gene have an excess of CD100 gene in all of their reproductive

cells and somatic cells. Homozygote animals with introduced CD100 genes in both homologous chromosomes can be acquired and all of the progeny bred through the generations so as to have an excess of CD100 gene by breeding the above male and female animals. The progeny obtained in this way are also included in the animals of the present invention.

The transgenic nonhuman animals with DNA that incorporates an exogenous CD100 gene or mutated gene thereof obtained in this way are nonhuman animals with enhanced T cell reactivity and elevated T cell interferon gamma production capacity and growth capacity (example 11 discussed below).

Nonhuman animals with a normal CD100 gene express a high level of normal CD100 gene and finally develop hyper-CD100 syndrome due to accelerated function of the endogenous normal CD100 gene. Therefore, they can be used as pathology models. For example, mechanisms of onset of hyper-CD100 syndrome and various diseases associated with CD100, e.g., diseases caused by abnormal antibody production or excessive antibody production (e.g., atopic asthma, allergic rhinitis, atopic dermatitis, allergic bronchitis, pulmonary aspergillosis, parasitic disease, Kimura's disease, hyper-IgE syndrome, Wiskott-Aldrich syndrome, thymic aplasia, Hodgkin's disease, liver cirrhosis, acute hepatitis, chronic rheumatoid arthritis, insulin-dependent diabetes, systemic erythematosus, scleroderma, infertility, endometriosis, autoimmune thyroid disease myasthenia gravis [sic], Hashimoto's disease, Basedow's disease, pernicious anemia, Addison's disease, male infertility, multiple sclerosis, Goodpasture syndrome, pemphigus, pemphigoid, myasthenia gravis, ophthalmia of the lens, sympathetic ophthalmia, autoimmune hemolytic anemia, idiopathic thrombocytopenia, autoimmune leukopenia, Felty syndrome, autoimmune lymphocytopenia, ulcerative colitis, Sjogren's syndrome, systemic autoimmune disease, primary biliary liver cirrhosis, lupoid hepatitis, etc.) and methods of

treating these diseases can be studied using normal CD100 transgenic animals.

Animals to which an exogenous normal CD100 gene has been transferred have symptoms of elevated free CD100. Therefore, they can be used to screen therapeutic drugs for the aforementioned CD100-related diseases. For example, inhibitors can be screened by inoculating the aforementioned mice with an exogenous antigen such as dinitrophenyl ovalbumin and measuring whether or not the antibody titer to the exogenous antigen drops due to appropriate administration of the test compound. More specifically, it is also possible to measure the level of cytokines in the blood such as interferon gamma that are believed to rise in autoimmune disease, etc.

On the other hand, nonhuman animals with an exogenous abnormal CD100 gene can be bred through the generations in an ordinary rearing environment as animals that possess said CD100 gene after confirming by breeding that they hold the CD100 gene stably. The target CD100 gene can also be used as a raw material incorporated into the aforementioned plasmids. The DNA construct with the promoter can be produced by ordinary CD100 genetic engineering techniques.

Transfer of an abnormal CD100 gene in the fertilized egg cell stage assures its presence in all of the reproductive cells and somatic cells of the subject animal. The presence of the abnormal CD100 gene in the reproductive cells of the animals prepared after transfer of the CD100 gene means that all of the progeny of the animals prepared have abnormal CD100 genes in all of their reproductive cells and somatic cells. The progeny of animals of this type that have inherited the CD100 gene have abnormal CD100 genes in all of their reproductive cells and somatic cells. Homozygote animals with the introduced CD100 gene in both homologous chromosomes can be acquired and all of the progeny can be bred through the generations to have said CD100 gene by mating these male and female animals.

Nonhuman animals with an abnormal CD100 gene express a high level of the abnormal CD100 gene and finally develop symptoms of inappropriate CD100 syndrome due to inhibition of the function of the endogenous normal CD100 gene. Therefore, they can be used as pathology models. For example, abnormal CD100 transgenic animals can be used to analyze the mechanisms of onset of inappropriate CD100 syndrome and to study methods of treating this disease.

A concrete possibility of utilization is for animals expressing a high level of abnormal CD100 gene to serve as a model for explaining inhibition of normal CD100 function by abnormal CD100 (dominant negative effect) in inappropriate CD100 function syndrome. Since animals to which an abnormal exogenous CD100 gene has been transferred lose normal CD100 function, they can serve as models of animals with weakened resistance to infections and diseases caused by viruses (such as colds, influenza, AIDS, hepatitis, herpes, measles, varicella, foot-and-mouth disease, herpes zoster, erythema infectiosum, rubella, exanthema subitum, viral conjunctivitis, viral meningitis, viral pneumonia, viral encephalitis, Lassa fever, Ebola hemorrhagic fever, Marburg disease, Congo hemorrhagic fever, yellow fever, dengue fever, rabies, adult T cell leukemia (ATL), rotavirus infection, polio, mumps, etc.), infections and diseases caused by bacteria or fungi (such as bacterial food poisoning, bacterial diarrhea, tuberculosis, Hansen's disease, dysentery, typhoid fever, cholera, paratyphus, plague, tetanus, tularemia, brucellosis, anthrax, septicemia, bacterial pneumonia, dermatomycosis, etc.) and enhanced growth of cancers (oral cavity cancer, pharyngeal cancer, lip cancer, tongue cancer, gingival cancer, nasopharyngeal cancer, esophageal cancer, stomach cancer, small intestinal cancer, large intestinal cancer including colon cancer, liver cancer, gallbladder cancer, pancreatic cancer, nasal cavity cancer, lung cancer, bone cancer, soft tissue cancer, skin cancer, melanoma, breast cancer, uterine cancer, ovarian cancer, prostate cancer,

testicular cancer, cancer of the penis, urinary bladder cancer, kidney cancer, brain tumor, thyroid cancer, lymphoma, leukemia, etc.). They can also be utilized to screen drugs for the treatment of these diseases.

The screening method of the present invention using transgenic nonhuman animals with DNA that incorporates an exogenous CD100 gene or mutated gene thereof may be implemented in combination with the aforementioned screening method (ligand-receptor assay system) for compounds or salts thereof that change the binding property between CD100 and CD72. Specifically, the present invention proposes a screening method for compounds (particularly compounds that inhibit the binding property between CD100 and CD72) or salts thereof that change the binding property between CD100 or salts thereof and receptors thereof characterized by using transgenic nonhuman animals with DNA that incorporates an exogenous CD100 gene or mutated gene thereof.

Here, compounds that change the binding property between CD100 and CD72 as a result of primary screening by the ligand-receptor assay system are selected as candidate compounds and the preventative and therapeutic effects of said candidate compounds then tested by secondary screening using transgenic nonhuman animals with DNA that incorporates an exogenous CD100 gene or mutated gene thereof. Or, candidate compounds are selected by primary screening using a screening system that employs transgenic nonhuman animals with DNA that incorporates an exogenous CD100 gene or mutated gene thereof and compounds that change the binding property between CD100 and CD72 obtained subsequently by secondary screening by the ligand-receptor assay system may be selected as candidate compounds for the preventative and therapeutic drugs of the present invention.

Other possible uses of the aforementioned two types of CD100 transgenic animals include the following:

(1) Use as a cell source for tissue culture,

(2) Direct analysis of the CD100 gene or RNA in the tissues of CD100 transgenic animals or analysis of the

relationship with proteins specifically expressed or activated by CD100 by analyzing tissues with a high level of CD100 gene expression,

(3) Research on the functions of cells from tissues that are generally difficult to culture by using cells of tissues with the CD100 gene cultured by standard tissue culture techniques,

(4) Screening drugs to increase cell function by using the cells of (3) above, and

(5) To isolate and refine modified CD100 and to produce antibodies thereof.

The clinical symptoms of diseases associated with CD100, including inappropriate CD100 function syndrome, can also be investigated using CD100 transgenic animals. Detailed pathology findings can also be obtained in each organ in models of CD100-associated diseases, new therapeutic methods developed, and contributions made to the research and treatment of secondary diseases caused by this condition.

CD100 transgenic cells can be acquired using a protein-degrading enzyme such as trypsin after removing organs from CD100 transgenic animals and mincing them. They can then be cultured and cultured cells lines developed. These can serve as effective research materials for studying the relationships with specialization, differentiation, and growth of CD100-producing cells or the signal transmission structure thereof, investigating abnormalities thereof, and explaining CD100 and its effects.

An effective, rapid screening method for therapeutic drugs for said diseases can also be proposed using the test and assay methods described above to develop drugs for the treatment of diseases associated with CD100, including inappropriate CD100 syndrome, using CD100 transgenic animals.

Methods of CD100 gene therapy in diseases associated with CD100 can also be studied and developed using CD100 transgenic animals or exogenous CD100 gene expression vectors. Virus vectors such as retrovirus vectors,

adenovirus vectors, AAV vectors, and herpes virus vectors or membrane fused liposomes, for example, are used to study gene therapies.

Abbreviations of the bases and amino acids, etc. in this specification and drawings are based on the IUPAC-IUB Commission on Biochemical Nomenclature abbreviations and abbreviations commonly used in this field. Examples are given below. When amino acids can exist as optical isomers, the L-form is shown unless specifically stated otherwise.

DNA:	deoxyribonucleic acid
CDNA:	complementary deoxyribonucleic acid
A:	adenine
T:	thymine
G:	guanine
C:	cytosine
Y:	thymine or cytosine
N:	thymine, cytosine, adenine, or guanine
R:	adenine or guanine
M:	cytosine or adenine
W:	thymine or adenine
S:	cytosine or guanine
RNA:	ribonucleic acid
MRNA:	messenger ribonucleic acid
DATP:	deoxyadenosine triphosphate
DTTP:	deoxythymidine triphosphate
DGTP:	deoxyguanosine triphosphate
DCTP:	deoxycytidine triphosphate
ATP:	adenosine triphosphate
EDTA:	ethylenediamine tetraacetate
SDS:	sodium dodecyl sulfate
EIA:	enzyme imunoassay
Gly or G:	glycine
Ala or A:	alanine
Val or V:	valine
Leu or L:	leucine
Ile or I:	isoleucine
Ser or S:	serine

Thr or T:	threonine
Cys or C:	cysteine
Met or M:	methionine
Glu or E:	glutamic acid
Asp or D:	asparatic acid
Lys or K:	lysine
Arg or R:	arginine
His or H:	histidine
Phe or F:	phenylalanine
Tyr or Y:	tyrosine
Trp or W:	tryptophan
Pro or P:	proline
Asn or N:	asparagine
Gln or Q:	glutamine
PGlu:	pyroglutamic acid
Me:	methyl group
Et:	ethyl group
Bu:	butyl group
Ph:	phenyl group
TC:	thiazolydine-4-(R)-carboxamide group
Bom:	benzyloxymethyl
NMP:	N-methylpyrrolidone
PAM:	phenylacetamide methyl

The substituents, protective groups, and reagents frequently used in this specification are represented by the following abbreviations

Tos:	p-toluenesulfonyl
HONB:	N-hydroxy-5-norbornene-2,3-dicarboxyimide
Bzl:	benzyl
Cl_2-Bzl:	dichlorobenzyl
Z:	benzyloxycarbonyl
Br-Z:	2-bromobenzyloxycarbonyl
Cl-Z:	2-chlorobenzyloxycarbonyl
Boc:	t-butyloxycarbonyl
HOBT:	1-hydroxybenzotriazole
DCC:	N,N'-dicyclohexylcarbodiimide
TFA:	trifluoroacetic acid
Fmoc:	N-9-fluorenylmethoxycarbonyl

DNP: dinitrophenyl
Bum: tert-butoxymethyl
Tri: trityl
BSA: bovine serum albumin
CHAPS: 3-[(3-cholamido)dimethylammonio]-1-propane sulfonate
E64: (L-3-trans-carboxyoxirane-2-carbonyl)L-leucyl-agmatin
DNP-OVA: dinitrophenyl ovalbumin
DNP-BSA: dinitrophenyl bovine serum albumin
ELISA: enzyme-linked immunosorbent assay
EIA: enzyme immunosorbent assay
PBS: phosphate buffered saline
LPS: lipopolysaccharide
conA: concavalin A

The sequence numbers in this specification indicate the following sequences.

[SEQ ID NO: 1] shows the amino acid sequence of mouse CD100.

[SEQ ID NO: 2] shows the base sequence of mouse CD100.

[SEQ ID NO: 3] shows the amino acid sequence of human CD100.

[SEQ ID NO: 4] shows the base sequence of human CD100.

[SEQ ID NO: 5] shows the amino acid sequence of mouse CD72.

[SEQ ID NO: 6] shows the base sequence of mouse CD72.

[SEQ ID NO: 7] shows the amino acid sequence of human CD72.

[SEQ ID NO: 8] shows the base sequence of human CD72.

[SEQ ID NO: 9] shows the base sequence of the primer on the N terminal used to prepare for the mCD100-Fc described in reference example 1.

[SEQ ID NO: 10] shows the base sequence of the primer on the C terminal used to prepare for the mCD100-Fc described in reference example 1.

Examples

The present invention is explained in greater detail below through reference examples and examples. However, these do not limit the scope of the present invention.

Reference example 1: Isolation of CD100 with expression enhanced by CD40 stimulation

1×10^8 mouse B cell line WEHI231 cells were stimulated for 8 hours using anti-CD40 antibody amd HM40-3 (PharMingen). The total RNA was isolated by the guanidine isothiocyanate phenol method from unstimulated or stimulated cells and mRNA was purified using oligo(dT)-bonded magnetic beads (Promega). cDNA synthesis and subtraction cloning were performed using a PCR-Select cDNA subtraction kit (Clontech). The cDNA fragment produced by CD40 stimulation was inserted directly into a T/A vector (Invitrogen). The CD100 gene described in the Journal of Biological Chemistry 271, 33376-33381 (1996) was isolated by comparing the base sequences obtained.

The mCD100-Fc described in examples 1 and 3 below is a protein with solubilized human IgG1 Fc fused to mouse CD100. Specifically, secretion-type mouse CD100 cDNA was prepared from mouse CD100 cDNA extracted from WEHI231 cells that had been stimulated by CD40 by PCR using an oligonucleotide that combined a primer containing a sense-wise SalI site (gctgtcgactgtgtgcccgttgctgaaggcct) [SEQ ID NO: 9] and a primer containing an antisense-wise BamHI site (gacggatcctacttactttgctttgcttgcttgagatacaccgtcttctctga) [SEQ ID NO: 10]. A gene that expresses mCD100-Fc protein was made by inserting the SalI-BamHI fragment obtained into the SalI-BamHI fragment DNA fragment of a pEFBos human IgG1 Fc cassette. Transformants were prepared by inserting this gene into a P3U1 plasmacytoma by electroporation (conducted at 0.25 kV, 960 microFD using a Biorad Gene Pulsar). Specifically, 10^7 cells were transformed by pEFBos-mCD100-Fc plasmid DNA that had been cleaved by 50 μg of HindIII and pMC1neo vector that had been cleaved by BamHI. After culturing for 10 days in RPMI medium that contained 10% fetal calf serum and 0.3

mg/mL of G418, the colonies resistant to G418 were isolated and cloned. mCD100-Fc protein was purified from the culture broth by protein A Sepharose (Amersham Pharmacia).

The biotinylated mCD100-Fc described in example 1 below was obtained by bonding biotin to mC100-Fc by a biotinylation kit (Boehringer-Mannheim). The CHO cells expressing CD100 described in example 2 were transformants obtained by inserting the CD100 gene into CHO cells. They express CD100 protein. Specifically, the entire length of the CD100 cDNA was incorporated into a pEFBOS vector and inserted together with a pMC1neo vector into the CHO cells using LipofectaminePlus (Life Technologies). The G418-resistant cells were selected after 10 days in the presence of 0.3 mg/mL of G418.

Reference example 2: Isolation of molecular CD72 bonded with CD100

2B4 cells from C57BL/6 mice were cultured using RPMI1640 medium that contained 10% fetal calf serum. 1×10^6 cells/mL of 2B4 cells were then stimulated for 18 hours by 2 μg/mL of conA. The total RNA was isolated from the cells by guanidine isothiocyanate density gradient centrifugation and the mRNA selected from the total RNA using oligo(dT)-bonded magnetic beads (Promega). Double-stranded cDNA that contained oligo(dT) was synthesized using a SuperScriptII cDNA synthesis kit (Life Technologies). A BstXI adapter (Invitrogen) was added to the cDNA, followed by fractionation by 1% agarose gel electrophoresis. The cDNA of at least 1.0 kb was recovered and inserted into a pME18S vector that had been cleaved by BstXI. *E. coli* DH10B cells (Life Technologies) were transformed by electroporation (conducted at 2.5 kV, 25 μFD using a Biorad Gene Pulsar) using this inserted DNA. COS7 cells were transformed using Lipofectamine Plus using a plasmic obtained from the *E. coli* made from 2×10^7 independent clones. After 3 days of transformation, the cells were recovered and resuspended in a concentration of 5×10^6 cells/mL in PBS

that contained 5% fetal calf serum, 2.5 μg/mL Fc block, and 5 μg/mL biotinylated mCD100-Fc and allowed to stand for 1 hour on ice. The cells were then washed with cold PBS and suspended in PBS that contained Dynabeads with M-280 streptoavidin bonded to them. After 30 minutes in suspension, the cells were washed 10 times by cold PBS using a magnetic particle concentrator. The extrachromosomal plasmid DNA was extracted by the Hirt method (Proceeding of the National Academy of Sciences of USA, 84, 3365-3369 (1987)). The plasmid DNA was inserted into *E. coli* DH10B cells by electroporation (conducted at 2.5 kV, 25 μFD using a Biorad Gene Pulsar) and second, third, and fourth transformations carried out by protoplast fusion. The above extraction by magnetic force was repeated four times. A clear 1.4 kb band was found as a result. Analysis of the base sequence of this 1.4 kb cDNA clone found it to be the total length of mouse CD72 cDNA [SEQ ID NO:: 6].

The CHO cells expressing CD72 described in example 1 are transformants obtained by inserting a CD72 gene into CHO cells. They express CD72 protein. Specifically, a pME18S vector that incorporated CD72 was inserted together with a pMC1neo vector into CHO cells using Lipofectamine Plus (Life Technologies). The G418-resistant cells were selected after 10 days in the presence of 0.3 mg/mL of G418.

Example 1: Binding of CD100 and CD72

mCD100-Fc was biotinylated using a biotinylation kit. 10^6 control CHO cells and CHO transformants expressing CD72 were reacted with biotinylated mCD100-Fc (40 μg/mL) for 1 hour on ice in stain buffer (PBS containing 2% fetal calf serum, 0.02% sodium azide, 2 mM calcium chloride, and 1 mM magnesium chloride) that contained 5 μg/mL of Fc block (PharMingen) for analysis by flow cytometry. After washing with stain buffer, the cells were stained for 20 minutes by FITC-labeled streptoavidin (Becton Dickinson). The cells were then washed with stain

buffer and the cells bonded to the FITC-labeled streptoavidin analyzed by flow cytometer.

The results are shown in Figure 1. The graph on the left shows the control CHO cells and that on the right the CHO cells expressing CD72. The dotted line shows when mCD100-Fc was not added and the solid line when mCD100-Fc was added. The abscissa gives the fluorescence intensity per cell and the ordinate gives the relative cell count. The fluorescence intensity did not change even when biotinylated mCD100-Fc was added to the CHO cells on the left. This shows that CHO cells do not bind to biotinylated mCD100-Fc. The fluorescence intensity was stronger when biotinylated mCD100-Fc was added (solid line) than when it was not (dotted line) in the CHO cells expressing CD72 on the right. This illustrates that biotinylated mCD100-Fc binds to CD72 on the surface of CHO cells expressing CD72.

Example 2: Class switch-enhancing effect of mouse CD100

Resting B cells from the spleen of C57BL/6 mice prepared in a concentration of 1×10^5 cells/well were added together with 100 units/mL of anti-CD40 monoclonal antibody or IL-4 and p-formaldehyde-immobilized CHO cells expressing CD100 (2×10^4 cells/well) to flat-bottomed 96-well microtiter plates and cultured for 7 days. The production of IgM or IgG1 immunoglobulin was measured by ELISA. Specifically, the culture broth or control IgM or IgG was diluted using 0.1M carbonate buffer (pH 9.6). 100 µL was aliquoted into each well of an EIA 96-well immunoplate (MaxiSorp, Nunc) and adhered by standing overnight at approximately 4°C. After washing each well with buffer A (pH 7.0, 0.02M phosphate buffer that contained 0.15M NaCl), 100 µL of enzyme-labeled anti-IgM or IgG1 antibody solution that had been diluted by buffer B (pH7.0, 0.02M phosphate buffer that contained 0.1% BSA and 0.15M NaCl) was added and reacted at 25°C for another approximately 2 hours. Each well was then washed with buffer A and 100 µL of alkaline phosphatase substrate solution (1 mg/mL of phosphatase substrate (Sigma), 100

mM Tris (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$) was added and reacted for 30 minutes at 25°C. The 405 nm absorbance was measured using an automatic microplate colorimeter. The amount of antibody in each reaction solution was assayed by reading against a calibrationn curve of the absorbance and antibody content produced separately by adhering known quantities of IgM and IgG1.

The experiment compared the IgM and IgG1 levels (1) when only medium was added in the absence of insufficient CD100-expressing CHO cells, (2) when only medium was added in the presence of CD100-expressing CHO cells, (3) when anti-CD40 antibody (αCD40) and IL-4 were added in the absence of CD100-expressing CHO cells, and (4) when anti-CD40 antibody (αCD40) and IL-4 were added in the presence of CD100-expressing CHO cells. The results are shown in Figure 2. The abscissa shows the results of (1), (2), (3) and (4) from left to right. The ordinate shows the antibody level (unit: ng/mL) determined from the absorbance. The presence of CD100 (2) had no effect on antibody production of either IgM or IgG1 in comparison to the unadded control (1). Stimulation by anti-CD40 antibody and IL-4 (3) induced antibody production of both IgM and IgG1 in comparison to the unadded control (1). Stimulation by anti-CD40 antibody and IL-4 in the presence of CD100 (4) raised IgG1 production more than (3) despite a slight decrease in IgM production in comparison to when stimulated by anti-CD40 antibody and IL-4. This illustrates induction of what is called class switch, the phenomenon whereby the class of antibodies produced and secreted by B cells switches from IgM to IgG1.

Example 3: In vivo antibody production-enhancing effect of CD100

C57BL/6 mice were immunized by intraperitoneal inoculation of 100 μg of dinitrophenyl ovalbumin (DNP-OVA) prepared in aluminum [sic]. Following immunization, the animals were administered 200 μg/day of human IgG1 myeloma protein or mCD100-Fc for 10 days. Serum was

collected 6 and 10 days after DNP-OVA administration. The antibody titer of antibody specific to DNP was measured by ELISA using DNP-BSA. Specifically, the immunized mouse serum was diluted using 0.1M carbonate buffer (pH 9.6). 100 μL was aliquoted into each well of an EIA 96-well immunoplate (MaxiSorp, Nunc) that had been coated by DNP-BSA and adhered by standing overnight at 4°C. After washing each well with buffer A (pH 7.0, 0.02M phosphate buffer that contained 0.15M NaCl), 100 μL of alkaline phosphatase-labeled anti-mouse IgM or IgG1 antibody solution that had been diluted by buffer B (pH7.0, 0.02M phosphate buffer that contained 25% Blockase (Dainippon Seiyaku) and 0.15M NaCl) was added and reacted for another 2 hours at 25°C to bond to the anti-DNP antibodies adhered to the wells. Each well was then washed with buffer A, 100 μL of alkaline phosphatase substrate solution added and reacted for 30 minutes at 25°C. The 405 nm absorbance was measured using an automatic microplate colorimeter. The DNP-specific antibodies were determined.

The results are shown in Figure 3. The graph on the left shows the antibody titer to DNP contained in the serum 6 days after DNP-OVA administration. The graph on the right shows that in the serum 10 days after administration. The white block on the abscissa shows the antibody titer when IgG1 myeloma protein was administered and the black block that when mCD100-Fc was administered. The ordinate anti-DNP gives the antibody titer to DNP. 1/1000 of the antibody level to DNP contained in the serum of control mice 12 days after administration was taken as one unit. When CD100 (mCD100-Fc) was administered, the antibody titer on day 6 was more than three times higher than the antibody titer on day 6 when the control human IgG1 myeloma protein was administered and surpassed the antibody titer on day 10 when the control human IgG1 myeloma protein was administered. This illustrates that CD100 has an important role in the induction of antigen-specific antibody production.

Example 4: Creation of CD100 knockout mice

Phage clones that contained an approximately 12 kb CD100 genomic DNA fragment were isolated from a 129/SvJ mouse liver genome library (Stratagene) using two probes derived from the CD100 cDNA sequence (total length and 1/1200 bp). A 1.6 Kb portion of this DNA fragment that contains part of the first exon where the initiation codon is present was substituted by a neomycin-resistance gene (provided by the Mother-Child Health Center) (Cell, Vol. 51 (1987), 503-512). A herpes simplex virus thymidine kinase gene (HSV-TK) was also inserted downstream of the CD100 genome gene for negative selection (Nature, Vol. 336 (1988), 348-352) to construct the targeting plasmid DNA. 1×10^6 E14-1 embryonic stem cells were transduced by 50 μg of this targeting plasmid DNA by electroporation. The embryonic stem cells into which the gene had been introduced were double selected by G418 (0.4 mg/mL; Life Technologies) and ganciclovir (2 μM; Syntex). Embryonic stem cells of two clones that had undergone homologous recombination were identified as a result of selecting those among 1000 resistant colonies that had undergone homologous recombination on the CD100 gene by southern blotting.

Blastocysts from C57BL/6 mice (Shizuoka Laboratory Animal Center, 6-8 weeks old) were inoculated with embryonic stem cells from the CD100 modified clone and transferred to ICR surrogates (Shizuoka Agricultural Cooperative for Experimental Animals, 6-8 weeks old). Chimerism of the young was evaluated by the agouti-like color of the fur. Knockout mice were prepared by further mating male chimeras with C57BL/6 female mice. Knockout of the CD100 gene in the offspring produced was analyzed by southern blotting. Genome DNA was isolated from the tail of the mouse, digested by BamHI, and submitted to agarose gel electrophoresis. The phoresced DNA was transcribed to a nylon filter (Amersham Pharmacia) and hybridized overnight with a radio-labeled probe (CD100 promoter region, 0.2 Kb). The filter was submitted to

autoradiography after washing for 1 hour at 65°C in 0.1 x SSC, 0.1% SDS.

The results are shown in Figure 4. A shows the CD100 wild gene, genetic map of the CD100 targeting vector, and CD100 genetic map when recombination was assumed to have occurred, from top to bottom. The exon in the 5' non-translation region is shown by a gray box and the exon of the translation region by a black box. B shows the site of cleavage by the BamHI restriction enzyme and E the site of cleavage by the EcoRI restriction enzyme. Neo represents the neomycin resistance gene, HSV-TK the herpes simplex virus thymidine kinase gene, and the arrow the direction of transcription of these genes. The probe shows the location of the probe used in southern blotting. When southern blotting was performed by BamHI cleavage, the gene length bonded to the probe was estimated to be 2.6 Kb in the wild gene and 1.2 Kb in the recombinant.

B shows the results of wild (+/+), heterozygote (+/-), and mutated gene homozygote (-/-) gene type analyzed by southern blotting. Only a 2.6 Kb fragment is found in the wild type, two fragments of 2.6 Kb and 1.2 Kb in the heterozygote, and only a 1.2 Kb fragment in the homozygote of the mutated gene. Since the gene length bonded to the probe was estimated to be 2.6 Kb in the wild gene and 1.2 Kb in the recombinant, this demonstrates that the mice prepared are knockout mice with the CD100 gene locus substituted to the anticipated length.

C shows the results of confirmation that the knockout mice do not express the CD100 molecule on the cells. Analysis was performed by flow cytometry after double staining spleen cells prepared from wild mice (+/+) and CD100 knockout mice (-/-) by biotinylated mouse CD100 antibody/FITC-labeled streptoavidin and phycoerythrin-labeled anti-B220 (cell surface marker from mouse B cells) antibody. In the figure, the ordinate and the abscissa express the production levels of B220 and CD100 molecules, respectively, on the cell surface by logarithm as the fluorescence intensity per cell. CD100-

positive cells were found in the wild mice, but no CD100-positive cells could be found in the knockout mice. Therefore, the knockout mice were confirmed not to express the CD100 molecule on the cells.

Example 5: Analysis of CD5 surface antigen in lymphocytes from knockout mice and wild mice

Peritoneal cells were collected by washing the peritoneal cavity with phosphate buffer that contained 2% FCS and 10 U/mL heparin. Cell suspensions prepared from the peritoneal cavity or spleen were analyzed after double staining by FITC-labeled anti-B220 antibody and phycoerythrin-labeled anti-CD5 antibody (both by PharMingen). B220 is a cell surface marker of mouse B cells. CD5 is known as a marker associated with autoantibody production (Autoimmunity, Vol. 30 (1999), pp. 63-69).

The results are shown in Figure 5. +/+ shows wild mice and -/- shows CD100 knock-out mice. In the figure, the abscissa and the ordinate express the levels of production of B220 and CD5 molecules, respectively, on the cell surface by logarithm as the fluorescence intensity per cell. The two cell marker-positive cell fractions are shown in boxes in the figure. While the percentage of cells positive for both B220 and CD5 among peritoneal cells was 14.6% in wild mice, it decreased to 7.49% in knockout mice. While the percentage of cells positive for both B220 and CD5 among spleen cells was 1.5% in wild mice, it decreased even more to 0.93% in knockout mice. These results suggest that CD100 contributes to expression of the CD5 molecule. Therefore, if it were possible to inhibit the function of CD100, it might be possible to treat autoimmune diseases by suppressing CD5, the expression of which is said to be potentiated in autoimmune disease.

Example 6: Antibody production to TD (T cell-dependent) antigen

Eight-week-old CD100 knockout and wild mice (n = 4 or 5) were intraperitoneally immunized twice (second time after 28 days) (n = 5) by 100 μg of aluminum salt that contained NP-CGG (4-hydroxy-3-nitrophenylacetyl chicken γ-globulin labeled compound). Blood samples were taken before and 14, 21, 28, 35, 72 days after immunization. The total IgG with avidity for NP (nitrophenyl groups) and the IgG that binds at high affinity among it were each assayed by ELISA using plates that had been coated by NP_2-labeled bovine serum albumin (BSA labeled by 12 4-hydroxy-3-nitrophenylacetyl groups) and NP_2-labeled bovine serum albumin. 96-well microplates (Nunc) were coated by NP_{12}-labeled bovine serum albumin or NP_2-labeled bovine serum albumin overnight at 4°C. After washing, 200 μL/well of blocking solution (50 mM Tris-HCl (pH 8.1), 1 mM $MgCl_2$, 0.15M NaCl, 1% BSA, 0.05% Tween 20) was added and allowed to stand for 1 hour at room temperature. Specimens of 100 μL/well diluted by blocking solution were allowed to stand for 1.5 hours at room temperature. After washing three times by PBS that contained 0.05% Tween 20, alkaline phosphatase-labeled goat antimouse IgG1 antibody was added. Following washing 1 hour later, phosphatase substrate (Sigma) was added and detection performed by the 405 nm fluorescence.

The results are shown in Figure 6. A shows the changes over time in NP_{12}-labeled bovine serum albumin-bindable IgG. B shows the changes over time in NP_2-labeled bovine serum albumin-bindable IgG. C shows the changes over time in the ratio of NP_2-labeled bovine serum albumin bindable-IgG and NP_{12}-labeled bovine serum albumin-bindable IgG. The ordinate shows the antibody levels in A and B. It shows the ratio thereof in C. The abscissa shows the days elapsed after immunization, taking the day of the first immunization as day 0. O shows the results in CD100 knockout mice and Δ in wild mice.

As shown in Figures 6A and 6B, antibody production was decreased in knock-out mice in comparison to wild mice, indicating that antibody production capacity to TD (T cell-dependent) antigen was impaired in CD100 knockout

mice. Production of the high affinity antibody in particular was more significantly decreased than the low affinity antibody. As shown in Figure 6C, the NP_2/NP_{12} ratio increased over time in wild mice. This illustrates the maturation in the B cell group that produces the high affinity antibody with the passage of time. However, the increase in the NP_2/NP_{12} ratio in knockout mouse was less than in wild mice, showing damage to the mechanism of maturation into high affinity antibody production. Decreases in antibody production capacity to TD (T cell-dependent) antigen, i.e., decreases in TD reaction, are known to weaken resistance to infections, etc. (Immunodeficiency Review, Vol. 3 (1988), pp. 101-121). Therefore, administration of CD100 or compounds that enhance the effects of CD100 might serve as novel therapeutics for infections, etc.

Example 7: Loss of T cell reactivity in CD100 knockout mice

Eight-week-old wild mice and CD100 knockout mice were immunized by KLH (keyhole limpet hemocyanine) suspended in Freund's complete adjuvant, intraperitoneally when T cells were collected from the spleen and to the footpad when they were collected from nearby lymph nodes. Nine days after immunization, CD4-positive T cells were prepared from the spleen or nearby lymph nodes by CD4-labeled magnetic beads (Magnetic Cell Sorting, Mitenyi Biotec). 1×10^5 cells were stimulated for 3 days by varying concentrations of KLH in the presence of irradiated (3000 rad) wild mouse spleen cells (5×10^5). 2 μCi of tritium thymidine was added for 12 hours and the intracellular radioactivity measured when studying the cell growth capacity. The IL-4 (interleukin 4) and IFN-γ (interferon-γ) in the 3-day cell culture supernatant were measured by an ELISA kit (R&D system).

The results are shown in Figure 7. A shows a comparison of the growth capacity of CD4-positive T cells from the spleen due to KLH stimulation in wild mice and CD100 knockout mice. B shows a comparison of the growth

capacity of CD4-positive T cells from the nearby lymph nodes due to KLH stimulation in wild mice and CD100 knockout mice. O shows the results in wild mice and Δ in CD100 knockout mice. The ordinate gives the intracellular radioactivity as an indicator of growth capacity. The abscissa shows the amount of KLH added. C shows the growth capacity of CD4-positive T cells from the nearby lymph nodes due to KLH stimulation, a comparison of IL-4 and IFN-γ production capacity in wild mice and CD100 knockout mice and the effects of CD100 addition. 50 μg/mouse of mCD100-Fc was administered intravenously for 6 consecutive days beginning the day after immunization. The results obtained using 4 μg/mL of KLH are shown. +/+ and -/- on the abscissa show the wild mice and knockout mice. + represents addition of CD100-Fc and - absence thereof. The ordinate shows the growth capacity, IL-4 level, and INF level from left to right.

As shown in Figure 7A, the reactivity of spleen T cells to KLH was decreased in knockout mice in comparison to wild mice. As shown in Figure 7B, a further decrease in reactivity in lymph node T cells was found in knockout mice, with no reactivity encountered even when the amount of KLH was increased. As shown in Figure 7C, the IL-4 and INF-γ production capacity of CD4-positive T cells in knockout mice was remarkably lower than in wild mice, with almost no production of either factor found. Moreover, addition of soluble CD100 brought out recovery from the decreases in reactivity. This confirmed that these abnormalities in knockout mice are mediated by CD100.

Activation of antigen-presenting cells is reported to induce activation of antitumor T cells and lead to antitumor immunity. Elevated growth capacity and accelerated IL-4 and INF-γ production are found in antitumor T cells in this instance. The results of this example demonstrated that activation of antigen-specific T cells is significantly decreased in knockout mice based on all indicators consisting of growth capacity, IL-4 production capacity, and INF-γ production capacity. This

suggests that CD100 is intimately involved in the activation of antitumor T cells. In fact, CD72, which is a CD100 receptor, is expressed on antigen-presenting cells and activated T cells (Annual Thoracic Surgery [sic], Vol. 61: (1996), 252-258). Therefore, CD100 is hypothesized to be able to actualize antitumor activity by activating T cells by acting directly on these cells.

Example 8: Study of soluble CD100 and antisingle-stranded DNA antibody contained in MRL/lpr autoimmune disease model mouse serum

C57BL/6, Balb/c, MRL/n, and MRL/lpr mice were purchased from SLC (Shizuoka Laboratory Animal Center). Blood samples were taken from the ocular fundus of 16-week-old mice. Mouse fused protein of soluble CD100 and Flag was prepared by PCR from CD100 cDNA prepared from WEHI-231 cells that had been stimulated by anti-CD40 antibody. As primers, 5-gctgtcgactgtgtgcccgttgctgaaggcct [SEQ ID NO: 9] containing an SalI site was used as the 5' end sequence and
5-gacggatcctacttactttgctttgcttgcttgagatacaccgtcttctctga [SEQ ID NO: 10] containing a BamHI site and Flag sequence as the 3' end sequence. An SalI-BamHI fragment produced by PCR was incorporated into the SalI-BamHI part of a pEFBos human IgG1 Fc cassette. 50 mg of CD100-Flag plasmid DNA that had been cleaved by HindIII and 5 mg of a pMC1neo vector that had been cleaved by BamHI were introduced into 10^7 P3U1 plasmacytoma by electroporation. The cells with the gene introduced were selected by RPMI1640 medium that contained 10% fetal calf serum and 0.3 mg/mL of G418. The CD100-Flag protein was purified using anti-Flag antibody-labeled agarose (Sigma).

Sandwich ELISA was conducted as follows to detect the soluble CD100. Rat antimouse CD100 antibody (clone BMA-12, 5 μg/mL) was coated overnight at 4°C in 96-well microplates (Nunc). After washing, 200 μL/well of blocking solution (50 mM Tris-HCl (pH 8.1), 1 mM $MgCl_2$, 0.15M NaCl, 1% BSA, 0.05% Tween 20) was added and allowed to stand for 1 hour at room temperature. Specimens of 100

μL/well that had been diluted by blocking solution and standard samples (mouse fused protein of soluble CD100 and Flag) were allowed to stand for 1.5 hours at room temperature. After washing three times by PBS that contained 0.05% Tween 20, 2 μg/mL of biotinylated rat antimouse CD100 antibody (clone BMA-8) was added. Alkaline phosphatase-labeled streptoavidin (Sigma) was added 1 hour later. Phosphatase substrate (Sigma) was added after washing. The soluble CD100 molecule was detected by the 405 nm absorbance. Antisingle-stranded DNA was prepared as follows. Double-stranded DNA was obtained by treating bovine thymus DNA (Sigma) by S1 nuclease (Sigma). Single-stranded DNA was obtained by cooling after boiling the double-stranded DNA for 15 minutes. 5 μg/mL of single-stranded DNA was adhered to 96-well microplates. Mouse serum was added as was alkaline phosphatase-labeled antimouse IgG antibody (Southern Biotechnology). Monoclonal antibodies BMA-8 and BMA-12 were produced as follows. Animals were immunized by a total of four subcutaneous administrations of 100 μg of CD100-Fc at one-week intervals. The adjuvant used in immunization was Freund's complete adjuvant the first time and Freund's incomplete adjuvant the second and subsequent times. The same amount was also administered intravenously 5 days before sacrifice of the rats. B cells were prepared from the rat spleen and fused with myeloma cells. Clones BMA-8 and BMA-12 that produce antibodies to CD100 were selected from the fused cells. The clones were transplanted to the peritoneal cavity of rats and antibodies purified from the ascites.

The results are shown in Table 1.

[Table 1]

	Soluble CD100 (ng/mL)	Single-stranded DNA (absorbance)
C57BL/6 (n = 7)	<12	-
Balb/c (n = 8)	<12	-
MRL/n (n = 10)	<12	0.005

MRL/lpr (n = 20)	166±172	0.184±0.063

Table 1 shows the relationship between the mouse line and the amounts of soluble CD100 and single-stranded DNA. MRL/lpr mice are autoimmune disease model mice selected from MRL/n mice. Although the level of soluble CD100 in the serum was below the detection limit (12 ng/mL) in C57BL/6, Balb/c, and MRL/n normal mice, it was greatly elevated (166 ng/mL) in the MRL/lpr autoimmune disease mice that exhibit symptoms similar to those encountered in autoimmune disease. The level of antisingle-stranded DNA antibody that serves as an indicator of the level of autobody associated with autoimmune disease was similarly greatly raised in MRL/lpr mice in comparison to MRL/n mice, making it clear based on the level of antisingle-stranded DNA antibody as well that the MRL/lpr mice exhibit symptoms similar to those of autoimmune disease. These results demonstrate a significant possibility that abnormal production of CD100 participates in MRL/lpr autoimmune disease mice and animal experiments show that CD100 inhibitors are effective in autoimmune diseases such as chronic rheumatoid arthritis.

Example 9: Increases in soluble CD100 and autoantibody levels associated with aging in MRL/lpr mice

The levels of soluble CD100 (A) and antisingle-stranded DNA antibody (B) were measured using 8- to 20-week-old mice. Blood samples were taken from the ocular fundus. Sandwich ELISA was performed as follows to detect the soluble CD100. 96-well microplates (Nunc) were coated overnight at 4°C by rat antimouse CD100 antibody (BMA-12.5 μg/mL). After washing, 200 μL/well of blocking solution (50 mM Tris-HCl (pH 8.1), 1 mM $MgCl_2$, 0.15M NaCl, 1% BSA, 0.05% Tween 20) was added and allowed to stand for 1 hour at room temperature. Specimens of 100 μL/well diluted by blocking solution and standard samples (mouse fused protein of soluble CD100 and Flag sequence) were

allowed to stand for 1.5 hours at room temperature. After washing three times by PBS that contained 0.05% Tween 20, 2 μg/mL of biotinylated rat antimouse CD100 antibody (BMA-8) was added. Alkaline phosphatase-labeled streptoavidin (Sigma) was added 1 hour later. Phosphatase substrate (Sigma) was added after washing and the soluble CD100 molecule was detected by the 405 nm absorbance. Antisingle-stranded DNA was prepared as follows. Double-stranded DNA was obtained by treating bovine thymus DNA (Sigma) by S1 nuclease (Sigma). Single-stranded DNA was obtained by cooling after boiling the double-stranded DNA for 15 minutes. 5 μg/mL of single-stranded DNA was adhered to 96-well microplates. Mouse serum was added as was alkaline phosphatase-labeled antimouse IgG antibody (Southern Biotechnologies).

The results are shown in Figure 8. A shows the level of soluble CD100 in the serum and B the level of anti single-stranded DNA antibody in the serum. The abscissa gives the age of the mice. The ordinate of B shows the antibody level. Taking the number obtained from the serum of 22-week-old mice as 1, the number of each specimen represents the multiple magnitude. The level of soluble CD100 in the serum was below the detection sensitivity in 8-week-old MRL/lpr mice. However, it rose with age and reached 116±89 ng/mL at the age of 16 weeks. Similarly, the level of anti single-stranded DNA antibody that serves as an indicator of the advance of autoimmune disease also rose with age. It was judged based on these results that elevation of the level of soluble CD100 in the serum correlates over time with the state of advance of autoimmune disease in MRL/lpr mice. These results also suggest the strong possibility that abnormal production of CD100 participates in MRL/lpr autoimmune disease mice and demonstrated that CD100 inhibitors are effective in autoimmune diseases such as chronic rheumatoid arthritis.

Example 10. Loss of dendritic cell reactivity in CD100 knockout mice

Bone marrow cells were gathered from 8-week-old wild mice and CD100 knockout mice. Dendritic cells were prepared by culturing the bone marrow cells for 6 days in the presence of GM-CSF. 2×10^5 cells/well of dendritic cells were placed in 96-well plates. Antimouse CD40 antibody (HM-40-3 (PharMingen), 2 μg/mL) or LPS (lipopolysaccharide, 10 μg/mL) was added and cultured for 72 hours. The IL-12 in the culture supernatant was measured by ELISA (Amersham Pharmacia).

The results are shown in Figure 9. The results in wild mice are shown in white and the results in knockout mice by crosshatches. The amount of IL-12 is shown on the ordinate. IL-12 production rose and dendritic cells were activated when dendritic cells from wild mice were stimulated by anti-CD40 antibody or LPS. However, IL-12 production declined and dendritic cell activation was impaired when dendritic cells from knockout mice were used. Antigen-representing cells are reported to release activated IL-12 to induce activation of antitumor T cells and generate antitumor immunity (Nature, Vol. 393 (1998), pp. 413-414). The results of this example showed a marked decrease in dendritic cell activation in knockout mice. This suggests that CD100 participates intimately in the activation of dendritic cells. Therefore, CD100 is assumed to be able to actualize antitumor activity by activating dendritic cells.

Example 11. Promotion of T cell reactivity in CD100 transgenic mice

A construct that incorporated the total length of membrane-type mouse CD100 cDNA or that with the intracellular region deleted into an Eμ vector composed of an immunoglobulin V region heavy chain gene promoter, immunoglobulin heavy chain gene intron enhancer, and immunoglobulin κ chain enhancer was produced to express B cell-specific mouse CD100. Transgenic mice were prepared by introducing this gene fragment into the fertilized eggs of C57BL/6 mice.

Eight-week-old wild mice and CD100 knockout mice were immunized intraperitoneally with KLH (keyhole limpet hemocyanine, 10 μg/mouse) suspended in Freund's complete adjuvant. Nine days after immunization, CD4-positive T cells were prepared from the nearby lymph nodes by CD4-labeled magnetic beads (Magnetic Cell Sorting, Mitenyi Biotec). 1×10^5 cells were stimulated for 3 days by varying concentrations of KLH in the presence of irradiated (3000 rad) wild mouse spleen cells (5×10^5). 2 μCi of tritium thymidine was added for 12 hours and the intracellular radioactivity measured when studying the cell growth capacity. The amount of INF-γ (interferon-γ) in the 3-day cell culture supernatant was measured by ELISA (R&D system).

The results are shown in Figure 10. The graph on the left shows the INF-γ production and that on the right the growth capacity. Open circle shows the results in wild mice and closed circle shows those in CD100 knockout mice. The abscissa shows the amount of KHL added. The growth capacity is expressed using the intracellular radioactivity as an indicator.

As shown in Figure 10, the INF-γ production level and growth capacity of CD4-positive T cells rose in transgenic mice in comparison to wild mice. This demonstrated activation of specific T cells by KLH. Activation of antigen-presenting cells is reported to induce activation of antitumor T cells and create antitumor immunity (Nature, Vol. 393 (1998), pp. 413-414). Elevation of growth capacity and accelerated INF-γ production capacity in antitumor T cells are found in this case. The results of this example demonstrated elevation of both indicators, INF-γ production and growth capacity, associated with antigen-specific T cell activation in transgenic mice. This suggests that CD100 participates intimately in the activation of antitumor T cells. CD72, a CD100 receptor, is actually expressed on antigen-presenting cells and activated T cells (Annual Thoracic Surgery, Vol. 61 (1996), pp. 252-258). Therefore, CD100 is assumed to be able to actualize antitumor

activity by activating T cells by acting directly on these cells. The transgenic mice also appear to be in a state of hyperimmune reaction due to enhanced CD100 production. Therefore, they may serve as models of diseases such as immunodeficiency assumed to be caused by CD100 enhancement.

Industrial Applicability

The screening method for compounds or salts thereof that change the binding property between CD72 or salts thereof and CD100 or salts thereof of the present invention characterized by using CD72 or salts thereof and CD100 or salts thereof is useful as a screening method for CD72 agonists that can be used as drugs for the prevention or treatment of infections or diseases caused by viruses (such as colds, influenza, AIDS, hepatitis, herpes, measles, varicella, foot-and-mouth disease, herpes zoster, erythema infectiosum, rubella, exanthema subitum, viral conjunctivitis, viral meningitis, viral pneumonia, viral encephalitis, Lassa fever, Ebola hemorrhagic fever, Marburg disease, Congo hemorrhagic fever, yellow fever, dengue fever, rabies, adult T cell leukemia (ATL), rotavirus infection, polio, mumps, etc.), infections and diseases caused by bacteria or fungi (such as bacterial food poisoning, bacterial diarrhea, tuberculosis, Hansen's disease, dysentery, typhoid fever, cholera, paratyphus, plague, tetanus, tularemia, brucellosis, anthrax, septicemia, bacterial pneumonia, dermatomycosis, etc.), and cancer (oral cavity cancer, pharyngeal cancer, lip cancer, tongue cancer, gingival cancer, nasopharyngeal cancer, esophageal cancer, stomach cancer, small intestinal cancer, large intestinal cancer including colon cancer, liver cancer, gallbladder cancer, pancreatic cancer, nasal cavity cancer, lung cancer, bone cancer, soft tissue cancer, skin cancer, melanoma, breast cancer, uterine cancer, ovarian cancer, prostate cancer, testicular cancer, cancer of the penis, urinary bladder cancer, kidney cancer, brain tumor, thyroid cancer, lymphoma, leukemia, etc.) and CD72 antagonists that can

be used as drugs for the prevention and treatment of diseases caused by abnormal antibody production or excessive antibody production (such as atopic asthma, allergic rhinitis, atopic dermatitis, allergic bronchitis, pulmonary aspergillosis, parasitic disease, Kimura's disease, hyper-IgE syndrome, Wiskott-Aldrich syndrome, thymic aplasia, Hodgkin's disease, liver cirrhosis, acute hepatitis, chronic rheumatoid arthritis, insulin-dependent diabetes, systemic erythematosus, scleroderma, infertility, endometriosis, autoimmune thyroid disease myasthenia gravis [sic], Hashimoto's disease, Basedow's disease, pernicious anemia, Addison's disease, male infertility, multiple sclerosis, Goodpasture syndrome, pemphigus, pemphigoid, myasthenia gravis, ophthalmia of the lens, sympathetic ophthalmia, autoimmune hemolytic anemia, idiopathic thrombocytopenia, autoimmune leukopenia, Felty syndrome, autoimmune lymphocytopenia, ulcerative colitis, Sjogren's syndrome, systemic autoimmune disease, primary biliary liver cirrhosis, lupoid hepatitis, etc.)